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A STATISTICAL AND HISTOLOGICAL SURVEY OF METASTATIC CARCINOMA IN THE SKELETON

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PAST estimates of the frequency of the occurrence of skeletal metastases from primary carcinoma in other organs have shown some variation. Thus from necropsy figures, Pürckhauer (1929) estimated that 54·5 per cent of prostatic carcinomas metastasise to the skeleton while Boyd (1953) gave a corresponding figure of 70 per cent. Several authors (Schmorl, 1908 ; Blumer, 1909 ; Joll, 1923) and more recently Abrams, Spiro and Goldstein (1950) have emphasised that higher figures will be obtained as a result of more thorough necropsy technique. Copeland (1931) and Geschickter and Copeland (1949) point out the value of radiological examination of the entire skeleton and it is clear that necropsy figures, however meticulous the examination, are to some extent unreliable.

At the present time there is still not complete agreement as to the factors determining new bone formation and resorption in skeletal metastases. The classical theory of new bone formation advanced by von Recklinghausen (1891) postulated as the essential factor a local hyperaemia of the marrow surrounding the tumour embolus resulting in a reactive growth of fibroblasts, osteoid, and finally bone. This view was rejected by Axhausen (1909) and more recently by Milch and Changus (1956) while it receives no positive support from Pietrogrande (1956). It has been suggested (Assman, 1907 ; Walther, 1948) that necrotic bone, acting as a foreign body, is the stimulus to new bone formation but this hypothesis receives no support at the present time (Weese, 1956) and the statement by Kauffmann (1929) that cancer cells may function directly as osteoblasts is unacceptable.

The secretion of chemical stimulants by carcinoma cells as a basis of new bone formation, originally put forward as a theory by Goetsch (1906) and supported by Axhausen (1909), Zemgulys (1931) and Willis (1952) finds no great favour at the present time (Milch and Changus, 1956 ; Pietrogrande, 1956). The presence and mode of action of chemical stimulants cannot be proved by current histological methods and in the case of prostatic carcinoma the whole concept has unfortunately become linked (Willis, 1953) with the action of acid phosphatase. The presence of increased amounts of this enzyme in the blood in cases of prostatic carcinoma with skeletal metastases was demonstrated by Gutman and Gutman (1938)

but it is not clear why an enzyme, capable only of attacking monophosphoric esters, can be expected to produce calcified bone in the absence of preformed osteoid ; there is general agreement (Ham, 1950) that the process of bone formation involves the preliminary formation by osteoblasts of an organic matrix and the subsequent deposition within it of bone salt. The direct conversion of mesenchymal marrow cells into functioning osteoblasts was suggested by Axhausen (1909) and supported by Geschickter and Copeland (1949) and Milch and Changus (1956) ; for this hypothesis there is abundant histological evidence.

Several theories have also been advanced to explain the osteolytic action of carcinomatous metastases in the skeleton. The concept of " halisteresis " proposed by von Recklinghausen (1891) was completely rejected by Axhausen (1909) and a direct osteolytic action by cells derived from the stroma of the tumour was considered a factor of only little importance by Milch and Changus (1956). Lacunar resorption by osteoclasts was considered to be of only secondary importance by Goetsch (1906) although Axhausen (1909) regarded it as the main factor. Willis (1952) concedes the importance of lacunar resorption but states that stromal foreign body giant cells may be easily confused with true osteoclasts while Weese (1956) considers that giant cells are not necessary for bone resorption. Milch and Changus (1956) believe that all giant cells observed are of foreign body type and suggest that the mechanical effect of compression of bone by dense masses of tumour cells may be an important factor. As the result of a very detailed study Pietrogrande (1956) considers that there is no direct reaction between tumour and bone and that new bone formation and resorption are conditioned by the reaction of the mesenchymal marrow cells to the presence of the tumour.

In the present investigation no attempt will be made to assess the incidence of skeletal metastases from individual organs for reasons considered above. On the other hand an analysis of the frequency with which different types of primary carcinoma metastasise to the skeleton appears worth while in view of the extensive material available and since Bryson and Spencer (1951) in a comparable survey of bronchial carcinoma showed that there was a possibility of only slight error due to selection of the clinical material. An attempt will also be made to relate the bone changes to the histological structure of the skeletal metastases and to determine the relative importance of the various factors mentioned above in the pathogenesis of new bone formation and resorption.

METHOD OF STUDY

The histological files of this department contain records of 14,154 necropsies from 1946 to 1955 inclusive, amongst which there are 320 examples of skeletal involvement by secondary carcinoma. It is of interest that during the same period surgical material was obtained from only 20 cases of metastatic carcinoma in bone ; the latter are not included in the present study. Necropsy was complete in all but two of the cases and histological examination was made of the primary growth or some of the visceral metastases, and usually both, in all of the cases ; material was available from more than one bone in nearly 30 per ent of all cases. It is emphasised that although necropsies are performed by a small group of pathologists and the skeleton is not examined routinely in all cases, a standard technique is employed as used at the London Hospital with only minor modifications. An examination of the relevant parts of the skeleton is carried out if justified on

clinical or radiological grounds or if there is evidence of any bone lesion at necropsy, and care has been taken to exclude examples of direct skeletal involvement by carcinoma.

All bone material was decalcified in Kristensen's fluid (formic acid, 90 per cent, 202 ml.; sodium formate 43·0 g.; distilled water up to 1000 ml.) until the process was complete as judged by the flexibility of the tissue. Routine paraffin sections stained with haematoxylin and eosin were available in each case while sections stained by van Gieson's method for collagen were available from the more recent necropsies.

The site and nature, whether of compact lamellar or cancellous type, of each portion of bone examined histologically was noted and a quantitative estimate of the degrees of resorption and new bone formation was carried out in the following manner. Resorption restricted to partial destruction of trabeculae with complete destruction of only a few trabeculae was designated " + ", whereas the presence of more extensive areas of complete destruction was designated " ++ ". In the same way new bone formation restricted to the apposition of bone on existing trabeculae with the formation of only a few new trabeculae was designated " + "; extensive formation of new trabeculae was designated " ++ ". It was of great importance to distinguish between callus formation, the result of pathological fracture, and new bone formation of a reactive nature due to the presence of the tumour. This was accomplished by noting the presence or absence of cartilage formation and appreciable amounts of osteoid; the latter tissue may be easily identified in routine histological material completely decalcified by means of a buffered organic acid (Meyer, 1956). It was found that an excess of osteoid was associated with cartilage formation only in fracture callus; cartilage and an excessive amount of osteoid were not present in areas of purely reactive new bone formation. At first sight although somewhat arbitrary, this method of assessment worked well in practice and it was possible to assign without difficulty the changes observed to the different groups enumerated above. It was found that changes involving different bones in the same case were remarkably consistent and that the amounts of resorption and new bone formation never varied, except in a very few instances, by more than " + " in either direction. In cases where two or more bones were examined the more severe degree of observed change was recorded purely as a matter of convention. Strict histological critieria were applied before recording a complete absence of bony reaction so that the total number of such examples will, therefore, be correspondingly small and such a case as that reported by Simpson (1926*a*) with histological illustrations becomes unacceptable.

RESULTS

The distribution of the present series of cases according to the site of the primary tumour is shown in the first column of Table I while the distribution of the male and female cases is shown in the second and third columns. For the purpose of later discussion the findings in two comparable series of cases investigated by other authors are shown in the last two columns of the table. The detailed histological findings in the larger individual groups of cases composing the present series are set out below but before describing these some of the general histological findings, especially with regard to new bone formation and bone resorption will be mentioned.

TABLE I

Site of primary tumour	Archway series (Total)	Archway series (Male examples)	Archway series (Female examples)	Montefiore series (Total)	Walther's series (Total)*
Bronchus	124	99	25	52	87 (*d*)
Breast	56	1	55	122	86
Prostate	53	53	—	16	47
Stomach	33	21	12	13	38
Intestine	11	5	6	22 (*a*)	10 (*e*)
Kidney	10	7	3	8	16
Unknown	8	5	3	0	0
Liver, gall bladder and bile ducts	6	3	3	0	13
Bladder and renal pelvis	6	4	2	5 (*b*)	4
Thyroid	4	1	3	5	25
Pancreas	3	2	1	4	3
Oesophagus	3	3	0	2	31
Uterus	2	—	2	6 (*c*)	24 (*f*)
Tongue	1	1	0	2	1
Ovary	0	0	0	6	2
Larynx	0	0	0	1	0
Nasal cavity	0	0	0	0	1
Mouth	0	0	0	0	4
Pharynx	0	0	0	0	15 (*g*)
Testicles	0	0	0	0	3
Vagina	0	0	0	0	2
Skin	0	0	0	0	3
Others	0	0	0	8	0
Total	320	205	115	272	415

* Melanomas, carcino-sarcomas, sarcomas and teratoblastomas have all been excluded from this table.

(*a*) Rectum and colon combined.
(*b*) Bladder only.
(*c*) Body and cervix of uterus combined.
(*d*) Includes 5 examples of pleural endothelioma.
(*e*) Rectum and colon combined.
(*f*) Body and cervix of uterus combined.
(*g*) Includes paranasal sinuses, naso-pharynx, mesopharynx and hypopharynx.

New bone formation was found to occur in only two ways, firstly, by the apposition of new bone on existing trabeculae and Haversian systems (Fig. 1) and secondly, by the direct conversion of marrow cells into functioning osteoblasts (Fig. 2). Although necrotic bone foci were a frequent finding (Fig. 3) these could not be interpreted as an essential stimulus to new bone formation. It is emphasised that the examination of a section stained by van Gieson's method for collagen, where this was available, greatly facilitated the assessment of new bone formation.

Lacunar resorption by osteoclasts was the commonest finding in most areas of active bone resorption (Fig. 4). Nevertheless a direct osteolytic action by fibrous connective tissue cells derived from the stroma of the tumour was observed in many instances; such an effect was frequently produced by squamous-celled carcinomas (Fig. 5). Another frequent observation was the presence of masses of proliferating carcinoma cells in immediate contact with bone trabeculae undergoing resorption, and in such instances the direct effect of mechanical pressure appeared to be the operative factor (Fig. 6).

Although bone resorption was frequently present as a single entity it was exceptional to observe the occurrence of new bone formation without some degree of associated resorption. In general there was no significant variation between the histological structure of the primary tumours and that of their visceral and skeletal metastases.

Metastasis from carcinoma of the bronchus.—The present series is composed of 99 male examples including one carcinoma of the trachea and 25 female examples. In 10 cases the site of the primary tumour was not identified with certainty but it was considered on anatomical and histological grounds to be most probably bronchial in origin. These 10 cases have been included in the above total of 124 examples.

Extensive resorption (++) was present in 58 examples which were mostly undifferentiated trabecular polygonal-celled, epidermoid, or squamous-celled carcinomas. In this group only 2 examples were well differentiated adenocarcinomas and 8 were oat-celled carcinomas. A slight degree of resorption (+) was present in 37 examples which included 16 oat celled carcinomas. No change was recorded in 4 examples, each an oat-celled carcinoma (Fig. 7). A slight degree of new bone formation (+) was present in 8 examples of which 7 were oat-celled carcinomas.

Extensive new bone formation (++) was present in 6 examples which varied in their histological differentiation but the majority were adenocarcinomas Equal amounts of resorption and new bone formation occurred in 8 examples including 6 oat-celled carcinomas showing only slight degrees of bone change. In one case there was extensive new bone formation (++) and resorption (++) in 2 different bones and in the remaining 2 cases the material was insufficient for an accurate assessment of bone changes.

Metastasis from carcinoma of the breast.—The present series includes 55 female examples and one male example. Extensive resorption was present in 23 examples of which 18 were poorly differentiated trabecular polygonal-celled carcinomas showing little or no tubule formation while the remaining 5 examples were adenocarcinomas.

Slight degrees of resorption or of new bone formation were present in 12 examples but no significant histological distinctions could be made between these two groups. A complete absence of bone change was not recorded in a single instance.

Extensive new bone fomation was present in 13 examples including 8 adenocarcinomas (Fig. 8). Equal amounts of resorption and new bone formation occurred in 7 examples of which 5 were poorly differentiated, trabecular polygonal-celled carcinomas. In one case the material was insufficient for an accurate assessment of bone changes.

Metastasis from carcinoma of the prostate.—The present series includes 53 examples. Extensive new bone formation was present in 38 examples of which 30 were highly differentiated adenocarcinomas (Fig. 9), whereas the other 8 showed a more undifferentiated trabecular and solid acinar structure.

Slight degrees of new bone formation or of resorption were present in 10 examples. These included 5 highly differentiated adenocarcinomas, while the remaining 5 showed a more undifferentiated trabecular and solid acinar structure. There were no cases showing equal amounts of resorption and new bone formation, and a complete absence of bone change was not recorded in a single instance.

Extensive resorption was present in only 2 examples, both of which were poorly differentiated trabecular and solid acinar carcinomas.

Extensive new bone formation and resorption were present in 2 different bones in 2 cases (Fig. 10, 11); in each of these there was significant histological variation between the osteoclastic carcinoma and the one causing new bone formation, the latter having a definite adenocarcinomatous structure. In one case the material was insufficient for an accurate assessment of bone changes.

Metastasis from carcinoma of the stomach.—The present series includes 21 male examples and 12 female examples. Extensive resorption was present in 8 examples, of which 6 were undifferentiated spheroidal-celled carcinomas, while the other 2 were adenocarcinomas. Slight resorption was present in 7 examples, of which 2 were adenocarcinomas, while one contained numerous "signet ring" cells. Slight new bone formation was present in 3 examples, of which 2 were undifferentiated spheroidal-celled carcinomas containing numerous "signet ring" cells.

Extensive new bone formation was present in 9 examples, all of which were undifferentiated spheroidal-celled carcinomas, but numerous "signet ring" cells were present in 4 of them (Fig. 12). Equal amounts of resorption and new bone formation occurred in 5 examples, mostly undifferentiated spheroidal-celled carcinomas.

In one case an undifferentiated spheroidal-celled carcinoma with numerous "signet ring" cells produced extensive new bone formation in one bone; in another bone extensive new bone formation and resorption were associated. A complete absence of bone changes was not recorded in a single instance.

Metastasis from other sites.—The remaining groups are individually too small to allow definite conclusions to be drawn from a detailed histological analysis although further reference will be made to some of them in the following discussion.

DISCUSSION

In a search of the literature two other large and comparable necropsy series were found, firstly, that described by Walther (1948) reporting on material from the Pathological Institute, Zurich, and secondly, that described by Abrams *et al.* (1950) reporting on material from the Montefiore Hospital. The findings of these authors will be compared with those obtained in the present series and the groups of cases will be considered in the same order as they have been presented above, starting with the pulmonary group.

Although Adler (1912) found 57 examples of skeletal metastasis in a series of 374 cases of primary bronchial carcinoma and Luck (1950) gave a corresponding figure of 25 per cent the importance of bronchial carcinoma as a source of skeletal metastases is not generally appreciated. Many standard text-books do not stress this point while some authors (Geschickter and Copeland, 1949) give very low figures. The high figure of 38·8 per cent is the outstanding feature of the present survey and may be taken as representative for the County of London during the last decade. It reflects of course the high and increasing incidence of bronchial carcinoma in the population as a whole (Doll and Hill, 1950; Himsworth, 1957) and accounts for the overall preponderance of male cases in the present series. The low figures recorded in Walther's series (21·0 per cent) and in the Montefiore series (19·1 per cent) are remarkable; the latter figure may be explained by the fact that the Montefiore Hospital is reserved primarily for the chronic sick but

the former figure remains unexplained. Histological analysis of the present series shows that undifferentiated, trabecular polygonal-celled bronchial carcinomas are predominantly osteolytic whereas oat-celled carcinomas show usually only slight bone changes or a complete absence of bony reaction. The latter finding may be explained by the general lack of stromal reaction to an oat-celled carcinoma; this fact led to the comparatively late recognition by Barnard (1926) of these neoplasms as epithelial tumours. It is a fact of great importance that the oat-celled variety of bronchial carcinoma may frequently give rise to skeletal metastases showing little or no evidence of bony change.

In the case of mammary carcinoma there is close agreement between the figure in the present series (17·5 per cent) and the corresponding figure (20·7 per cent) in Walther's series, while the high figure (44·8 per cent) in the Montefiore series can be explained by the nature of that particular hospital. Histological analysis of the present series shows that undifferentiated polygonal-celled carcinomas are predominantly osteolytic. Four of the 5 adenocarcinomas producing extensive resorption of bone were in subjects over 70 years of age; in these 4 cases the osteolytic effect of the tumour was probably greatly accentuated by associated senile osteoporosis. It is of great interest that the predominating type of mammary carcinoma producing extensive new bone formation in its skeletal metastases is the adenocarcinoma. The definite association of an osteosclerotic tendency with this type of tumour and of an osteolytic tendency with the undifferentiated variety thus shows that the bony reaction is clearly related to the rate of growth of the tumour. It has already been shown by Scarff and Handley (1938) and Bloom (1950) that the undifferentiated variety of mammary carcinoma carries a worse prognosis, because of its more rapid growth, than does the more highly differentiated adenocarcinoma.

The surprisingly low figure of 16·6 per cent for prostatic carcinoma in the present series is higher than those recorded in Walther's series (11·3 per cent) and in the Montefiore series (5·9 per cent) but no obvious reasons for these discrepancies are apparent. A very high degree of histological differentiation with almost complete absence of cellular pleomorphism and mitotic activity is characteristic of many prostatic carcinomas; these features are most logically interpreted as representing a slow rate of growth and their correlation with a very marked osteosclerotic tendency is again a prominent feature in the present series. The two examples in which a significant variation in histological structure was associated with opposite bony reactions are of particular interest in this connection.

In the case of gastric carcinoma there is close agreement between the figure in the present series (10·3 per cent) and the corresponding figure (9·2 per cent) in Walther's series but the low figure (4·8 per cent) in the Montefiore series remain unexplained. There is no obvious reason for this discrepancy but the former figures are of interest since the importance of gastric carcinoma as a source of skeletal metastases was, until comparatively recently, unrecognised. Thus, Moore (1919) was unable to find a single example of sketelal metastasis in 1600 radiologically investigated cases of gastric carcinoma while Kaufmann (1929) found skeletal metastases in only 2·5 per cent of autopsies on 309 cases of gastric carcinoma. Histological analysis of the present series shows few significant features although the presence of large numbers of "signet ring" cells appears to predispose to new bone formation.

Although carcinoma of the kidney has always been considered a frequent

source of skeletal metastases, low figures are recorded in the three main series of cases under discussion. This finding reflects only the low incidence of renal carcinoma in the general population since high rates of metastasis have been reported by several authors (Albrecht, 1905; Symmers, 1917; Geschickter and Copeland, 1949; Willis, 1953). All the 10 examples in the present series of cases were osteolytic, a finding with which there is general agreement. The osteolytic tendency of renal carcinomas is most easily explained by the presence of a very vascular and active stroma since the clear-celled renal carcinoma must, on histological grounds, be considered a highly differentiated adenocarcinoma.

The extremely low figures for thyroid carcinoma in the present series (1·3 per cent) and in the Montefiore series (1·8 per cent) are remarkable and again reflect the very low incidence of thyroid carcinoma in the general population, while the higher figure (6·0 per cent) reported by Walther is at least partly explained on geographical grounds (Wynder, 1952). The general and quite erroneous view prevails that carcinoma of the thyroid is a frequent source of skeletal metastases; it is in some way connected with the interest shown in the concept of "benign metastasising goitre" introduced by Cohnheim (1876) and since then shown to be fallacious by several authors (Bérard and Dunet, 1921; Bell, 1924; Simpson, 1926*b*; Schaukowski, 1956).

In conclusion several points of interest emerge from the present study. The age of the cases was in general not a significant factor although it probably explained the presence of extensive bone resorption in the skeletal metastases of a small group of well differentiated mammary adenocarcinomas. The predominance of the male sex in the present series has already been mentioned in connection with the examples of bronchial carcinoma where the usually accepted ratio of four males to one female was observed.

EXPLANATION OF PLATES

FIG. 1.—Metastasis in vertebra from oat-celled bronchial carcinoma. There is apposition of new lamellar bone on the surfaces of existing trabeculae. × 60.

FIG. 2.—Metastasis in vertebra from bronchial adenocarcinoma. Extensive new bone formation has resulted from conversion of mesenchymal marrow cells into functioning osteoblasts. × 18.

FIG. 3.—Metastasis in sternum from prostatic carcinoma. Extensive necrosis has occurred in newly formed bone as shown by the disappearance of osteoblasts. × 60.

FIG. 4.—Metastasis in tibia from bronchial carcinoma. All the lacunae on the surfaces of both trabeculae are occupied by osteoclasts. × 60.

FIG. 5.—Metastasis in ilium from unknown source. The squamous-celled carcinoma has a very active fibrous stroma which is causing direct resorption of the bone trabecula. × 74.

FIG. 6.—Metastasis in rib from oat-celled bronchial carcinoma. The newly formed bone trabecula shows osteoid borders on two sides. On another side a cluster of proliferating oat cells has produced some resorption. × 250.

FIG. 7.—Metastasis in vertebra from oat-celled bronchial carcinoma. The bone shows a complete absence of reaction to the presence of the invading tumour. × 23.

FIG. 8.—Extensive new bone formation in vertebral metastasis from mammary adenocarcinoma. × 85.

FIG. 9.—Extensive new bone formation in vertebral metastasis from prostatic adenocarcinoma. × 74.

FIG. 10.—Well-differentiated metastasis in vertebra from prostatic carcinoma. Extensive new bone formation is associated with some degree of resorption. × 85.

FIG. 11.—More undifferentiated metastasis in rib from same case as shown in Fig. 10. Extensive resorption is associated with complete absence of new bone formation. × 85.

FIG. 12.—Extensive new bone formation in vertebral metastasis from "signet ring"-celled carcinoma of stomach. × 85.

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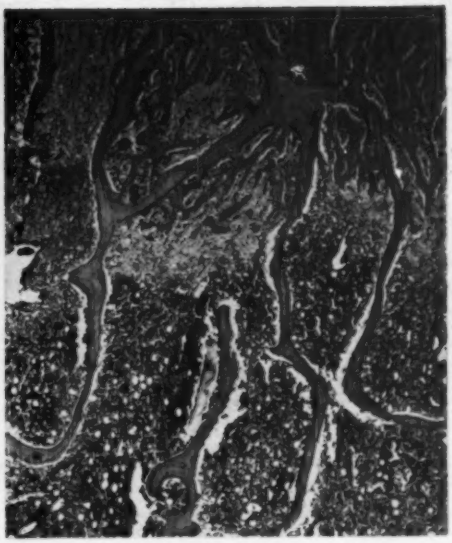

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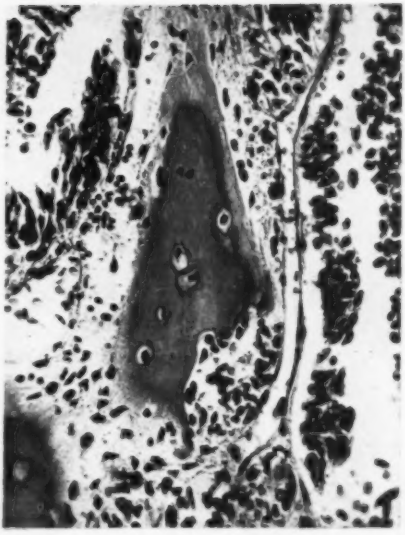

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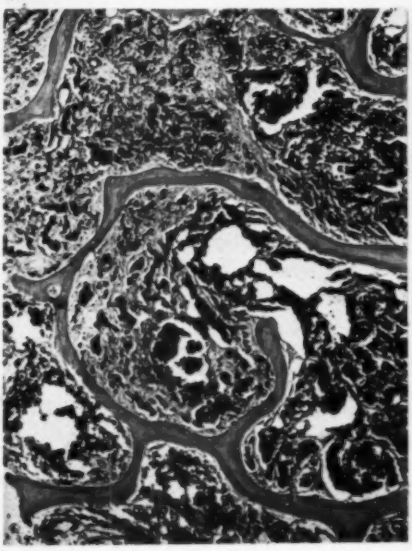

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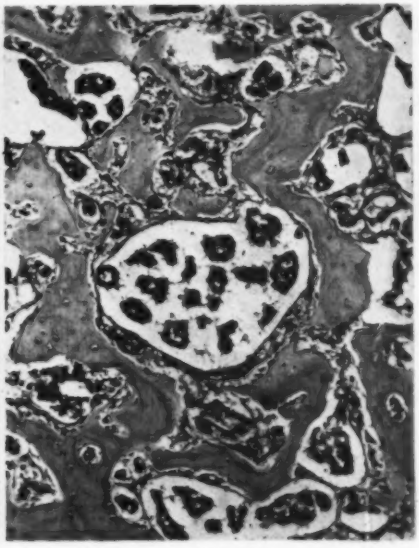

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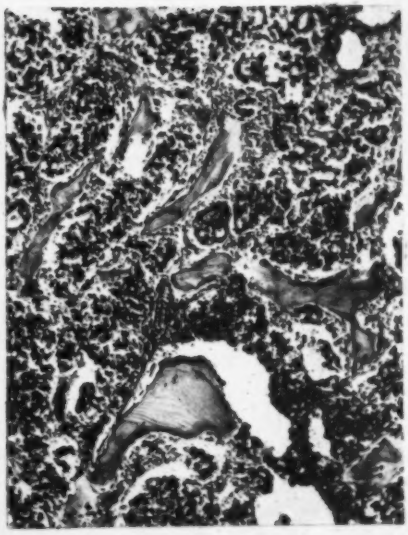

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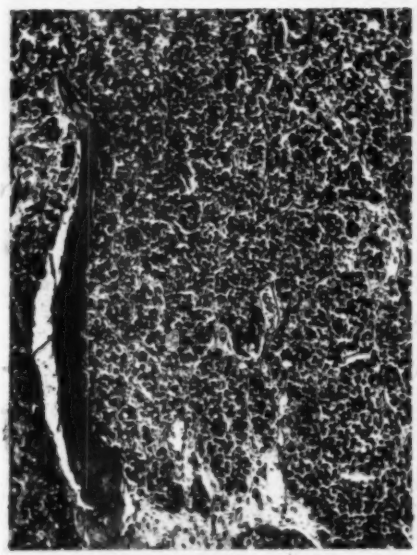

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Although malignant melanomas were excluded from the present study because of their controversial nature it is of interest that the histological files contain only one example with skeletal metastases during the period under review. This finding presumably again reflects the great rarity of this condition.

No attempt has been made to assess the frequency of pathological fracture in the series as a whole except to distinguish clearly between an osteosclerotic reaction to a tumour and new bone formation in callus resulting from a pathological fracture. The latter was, nevertheless, frequently observed in many examples of prostatic carcinoma producing extensive new bone formation in their skeletal metastases. This may be explained by consideration of the normal architecture of a bone which is adapted to provide maximum strength with minimum weight. It is clear that destruction of even small numbers of trabeculae with replacement by equal or greater numbers of irregularly arranged trabeculae must result in weakening of the bone and ultimately in pathological fracture.

SUMMARY

A series of 320 necropsies, in which the skeletal system was involved by secondary carcinoma, has been analysed in terms of the primary site of the neoplasm and a large proportion (124 cases) was attributed to primary carcinoma of the bronchus.

Although gastric carcinoma is not generally considered a frequent source of skeletal metastases the present series included 33 examples. The very low figures for renal and thyroid carcinomas are of great interest and reflect the low incidence of these conditions in the general population.

Osteoclastic erosion was the main operative factor in resorption of bone but a direct osteolytic action was frequently exerted by the stromal cells of the tumour. In many instances resorption of bone appeared to be the result of direct pressure by masses of proliferating epithelial cells. Osteosclerosis was produced by the apposition of new bone on existing trabeculae and by the formation of new trabeculae as a result of metaplasia of marrow cells.

A complete absence of bony reaction or only a slight reaction was frequently observed in the case of the oat celled variety of bronchial carcinoma, while a relationship between osteosclerosis and a high degree of histological differentiation could be established in the case of mammary and prostatic carcinoma.

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METASTASES IN PITUITARY TISSUE REMOVED AT HYPOPHYSECTOMY IN WOMEN WITH MAMMARY CARCINOMA

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HORMONE control and endocrine surgery are now accepted methods of palliating advanced carcinoma of the breast. Since anterior pituitary hormones such as prolactin and growth hormone are implicated in physiological breast development, in addition to ovarian and adrenal hormones, it was logical to study the clinical effect of hypophysectomy. Luft and Olivecrona (1953), and Pearson, Ray, Harrold, West, Li, and Maclean, (1954) confirmed that breast carcinoma of the so-called hormone-dependent type might regress after pituitary ablation.

MATERIAL

At the Royal Free Hospital, 70 patients with carcinoma of the breast or prostate, or malignant melanoma, have undergone hypophysectomy (Baron, Gurling and Radley Smith, 1958). The pituitary is approached through a right fronto-temporal osteo-plastic flap and the gland parenchyma removed in pieces by forceps, curettes, and suction. The tissue sent for histological study is therefore fragmented and does not as a rule comprise the whole gland.

According to Wyeth (1934), the weight of the pituitary gland in a group of women with cancer (from various sites) is greater than in the healthy individual, ranging from 0·77 g. to 1·05 g. It is usual to obtain about 0·4 g. for examination, but in spite of this hypophysectomy has been found at autopsy to be virtually complete in the majority of cases. The specimens were studied to confirm that anterior lobe tissue had in fact been removed, to find any variation in the appearances of the anterior lobe cells, and lastly to detect the presence of metastases. The fragments were usually fixed in formol saline, and occasionally in Carnoy's or Helly's fluids, and stained with haemotoxylin and eosin or Mallory's trichrome. We do not claim to have made exhaustive studies of these pituitary fragments since only representative fragments were cut, and the proportion with metastatic deposits may well be higher than we have found.

RESULTS

Adequate material was obtained from 44 of a consecutive series of 50 patients undergoing hypophysectomy because of breast cancer. Among the 6 excluded were 2 patients in whom the operation was virtually limited to pituitary stalk section, 2 in whom only posterior lobe tissue was obtained, and 2 the specimens from whom were lost.

Metastatic deposits were found in 11 of the 44, an incidence of 25 per cent. The clinical findings, histological appearances and response to operation are

TABLE I.—*Summary of Clinical Findings and Histological Appearances of the Primary Breast Growth and the Pituitary Metastases*

(Under " clinical response ", ++ = regression of growth ; + = arrest.)

				Distribution of metastases					Histological appearances			
No.	Patient	Age (yrs.)	Duration of disease (mnths.)	Local	Bones	Lung or pleura	Liver or peritoneum	Adrenals	Pituitary metastases	Breast growth	Clinical response	Remarks
1	D. S—	44	24	+	+	0	+	+	Anaplastic carcinoma infiltrating anterior lobe	Anaplastic carcinoma	0	Metastases found in pituitary fossa at autopsy.
2	E. P—	48	22	0	+	+	+	0	Polygonal cell carcinoma in posterior lobe	Undifferentiated scirrhous carcinoma	++	No growth in fossa two years later (^{198}Au seeds)
3	H. W—	51	40	+	+	0	0	—	Hyperchromatic undifferentiated ca. cells scattered through anterior lobe	Undifferentiated scirrhous carcinoma	++	Alive and well 2 years later (^{198}Au seeds).
4	H. E—	56	13	0	+	0	+	0	Undifferentiated ca. cells clumped in anterior lobe	Undifferentiated scirrhous carcinoma	+	Metastases found in pituitary fossa at autopsy (^{198}Au seeds).
5	G. C—	48	36	0	+	+	0	0	Moderately differentiated adeno- and papillary ca. in anterior lobe	Anaplastic with attempt at acini formation	++	Alive and well 15 months later (^{198}Au seeds).
6	A. N—	65	108	+	+	+	0	—	Trabeculae of small undifferentiated ca. cells in anterior pituitary	Adenocarcinoma	0	No autopsy (^{198}Au seeds).
7	V. P—	46	9	+	+	+	0	—	Poorly differentiated anaplastic ca. in anterior lobe	Adenocarcinoma—poorly differentiated in deeper areas	++	Alive and well 1 year later (^{198}Au seeds).
8	D. M—	32	36	+	+	+	0	—	Scattered foci of anaplastic carcinoma cells in anterior lobe	Undifferentiated small duct carcinoma	++	Alive and well 9 months later (^{198}Au seeds).
9	F. C—	54	6	+	0	0	+	0	Undifferentiated ca. in posterior lobe	Undifferentiated carcinoma	0	No autopsy (^{198}Au seeds).
10	B. J—	62	60	+	+	+	0	+	Undifferentiated carcinoma in anterior lobe tissue	Undifferentiated carcinoma	0	No carcinoma tissue in pituitary fossa at autopsy (^{198}Au seeds).
11	M. H—	52	10	+	0	+	0	—	Undifferentiated ca. scattered through anterior lobe tissue	Undifferentiated carcinoma	0	No autopsy.

summarised in Table I. In some instances difficulty was experienced in differentiating between anaplastic carcinoma cells and the hyperchromatic cells which sometimes appear as artefacts in the anterior pituitary lobe, and only those cases in which the distinction was obvious were included (Fig. 1, 2). The proportion of carcinomas that were anaplastic in the patients with pituitary metastases was the same as in the whole series of patients who underwent hypophysectomy. In all but two instances the metastases were present in anterior lobe tissues.

There do not appear to be any significant differences between the histological appearances of the primary breast growth and the pituitary metastases. In 8 cases both were histologically similar, in one the pituitary deposits appeared better differentiated, and in two they were less well differentiated than the primary growth. The potential local effects of the pituitary hormones do not seem to have induced either differentiation or anaplasia of the metastatic cancer tissue in the gland parenchyma. When analysed according to clinical response, of the 10 patients with pituitary metastases who survived operation, 4 (40 per cent) failed to improve and 6 (60 per cent) had a good response with arrest or regression of the tumour. On the other hand, there were 7 (57 per cent) of the 30 survivors without metastases who did not improve, and 13 (43 per cent) who responded (Table II).

TABLE II.—*Clinical Response to Hypophysectomy in Relation to the Presence or Absence of Metastases in the Pituitary Gland*

	Improved	No response	Total
Metastases in pituitary	6 (60%)	4 (40%)	10 (100%)
No metastases in pituitary	13 (43%)	17 (57%)	30 (100%)

The proportion improved after operation is slightly higher in patients with metastases in the pituitary, 60 per cent, against 43 per cent in the remainder. These figures are too small to permit valid conclusions.

DISCUSSION

All these patients were suffering from widespread metastatic breast carcinoma with involvement of the bones, lungs and liver, and the chance of haematogenous spread to any organ was therefore high. Yet adrenal metastases were found in no more than 2 of the 6 patients in whom these glands were examined. It is well known that metastatic deposits may be found in the pituitary, and were described by Simmonds (1913) and Schmorl (1914) in patients with carcinoma of the breast. Wyeth (1934), however, found metastases in the pituitary in only 5 (6·2 per cent) of his series of 80 post mortem examinations on patients dying of malignant disease. These authors, as well as Willis (1952), emphasise that the posterior lobe is almost invariably involved and that the anterior lobe usually escapes. Piney and Coates (1924), for example, described a patient suffering from mammary carcinoma who developed diabetes insipidus because of destruction of the pars nervosa, and found other cases recorded in the literature. We have found a preponderance of metastases in the anterior lobe, which number 9 (82 per cent) against 2 (18 per cent) in the posterior lobe. The pituitary was as often the site of metastases as the adrenals, which Glomset (1938) found to be invaded in 58 per cent of his autopsy series. According to Sheehan and Summers (1949), however, pituitary insufficiency (Simmonds's disease) is unlikely to arise from involvement

of the anterior lobe by haematogenous metastases. In none of our patients did either the anterior or posterior lobes appear to be destroyed to any significant degree.

It is impossible to say whether the high incidence of metastases in the pituitary in this group is unusual. There are no reports concerning material comparable to ours, but Kennedy, French and Peyton (1956) have reported metastases in one of their 28 hypophysectomised patients. Now that surgical specimens are available for examination, there seems little doubt that metastatic deposits in the anterior pituitary are much more common than has been thought. We cannot at this stage say whether or not there is any relation between the presence of such metastases and the degree of hormone dependence exhibited by the primary growth, but it seems unlikely.

SUMMARY

Specimens of pituitary tissue from 44 women with advanced carcinoma of the breast undergoing hypophysectomy have been examined histologically. Metastases were found in 11 (25 per cent), the anterior lobe being more frequently involved than the posterior lobe. These findings have been reviewed in relation to the post-operative clinical response.

We are greatly indebted to Mr. E. J. Radley Smith who performed the operation of hypophysectomy on all these patients, to the British Empire Cancer Campaign for their generous financial support, and to Professor K. R. Hill for his advice and interest.

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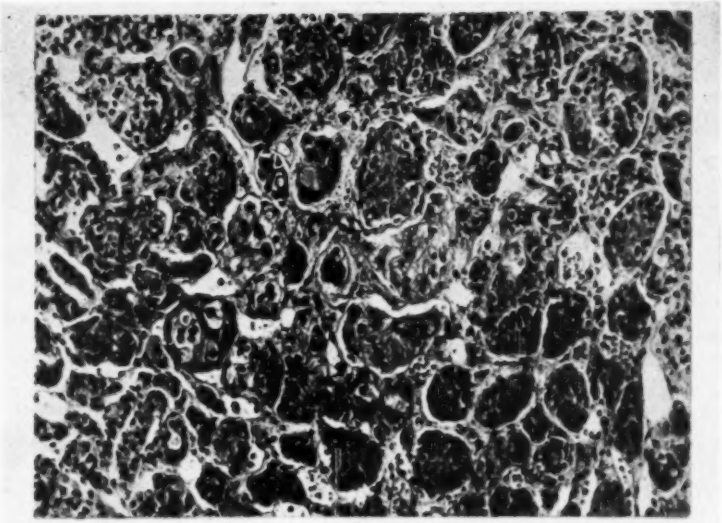
EXPLANATION OF PLATE.

Fig. 1.—Case 8. Focus of anaplastic carcinoma cells in anterior pituitary lobe. Haematoxylin and eosin. × 160.

Fig. 2.—Case 11. Focus of anaplastic carcinoma cells infiltrating anterior pituitary lobe. Haematoxylin and eosin. × 160.



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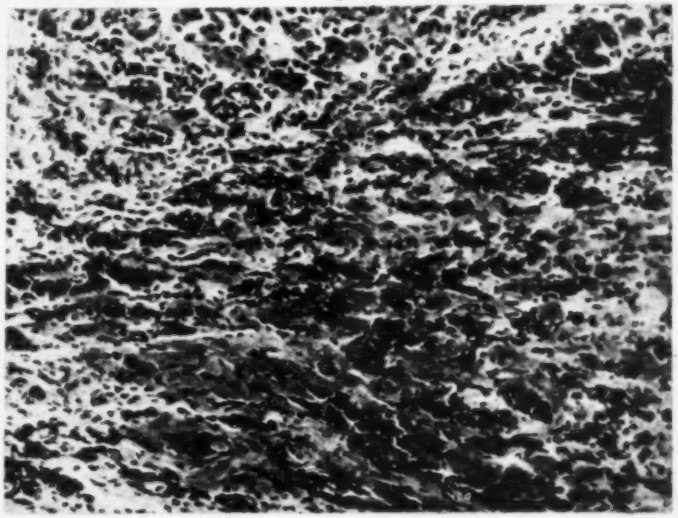

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CIRRHOSIS AND PRIMARY LIVER CARCINOMA IN UGANDA AFRICANS

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THERE is now mounting evidence to support the view that hepatocellular carcinoma occurs with undue frequency in the indigenous population of the African Continent. The relationship of cirrhosis to the development of carcinoma is not precisely established and studies to elucidate the nature of the relationship in Africa have only been undertaken in recent years. This report is based on a study of cases in the files of the Department of Pathology of the Makerere College Medical School on which autopsy examinations were performed at Mulago Hospital, Kampala during the years 1946–57. Not all the cases of cirrhosis or of carcinoma encountered in this period could be utilised, but the sample studied was representative and unbiased, being selected because of the ready availability of material and the mildness of the post-mortem changes. Because the sample is incomplete the statistical aspects are not included in the report. In any event they, as frequency studies, would soon be superseded by the results of an incidence rate study now being made. Preliminary results (Knowelden, 1957) suggest that the incidence of liver cancer in Uganda males is two and a half times that of a corresponding Danish population but that an elevated frequency in Uganda females could not be shown. Relative to other malignant tumours, liver cancers ranked fourth, being exceeded only by those of the uterine cervix, skin and penis (Davies, 1957). Carcinoma of the liver thus is an important and major neoplastic disease in Uganda. Most cancers arise in cirrhotic livers so that the cirrhosis also demands study.

This report will attempt to characterise the pathological features both of cirrhosis and of cancer in Uganda Africans. Because the ultimate objective is to find aetiological factors, special attention was paid during this study to the histopathogenesis of the lesions and to possible causative agents of both diseases. It is known from Africa (Berman, 1951) and other countries (Steiner, 1954) that where the frequency of liver cancers is high, the disease often comes on early in life. The higher yield of tumours and the possible shortened induction time suggest that the aetiological factors are intensified. A study of the exaggerated situation might reveal cause-and-effect relationships not apparent where the frequency is low.

Some special problems given consideration included the following :—

1. Is the high incidence of liver cancer due merely to more cases of the usual morphological types or do new varieties enter the picture ?

2. Is there anything peculiar or different about the cirrhosis, which is so often and so quickly associated with cancer ?

3. Is there anything discernible in the liver of the African which would explain the high frequencies of cancer and cirrhosis ? This thought arises from the observations of Dorn (1955) that the Negro and Caucasoid in the United States do not differ from each other appreciably either in the incidence of cirrhosis or of cancer.

4. Is the aetiology of either disease apparent or suggested by the morphological or the cytopathogenic picture ?

As a basis for contrast on some of these points, one of the authors (P. E. S.) has studied 137 cases of liver cancer in the U.S.A. [100 in Los Angeles (Edmondson and Steiner, 1954) and 37 in Chicago] and about 400 cases of cirrhosis occurring in some 10,000 autopsies on record in Chicago.

Basis of the study

This study is based on 70 cases of primary liver carcinoma and of 100 cases of advanced cirrhosis. In 50 of the cirrhotic livers a primary carcinoma was present. An additional 12 cases in which mild cirrhosis was an incidental post-mortem finding were also studied for the light they shed on histogenesis, but the main basis for the report of cirrhosis is the 100 advanced cases. About 100 other livers showing various other lesions, including hepatitis, kwashiorkor, acute necrosis, fatty change, schistosomiasis and other changes were studied and mention will be made of some of these cases later. All were native Africans *sensu stricto*, from a variety of tribes. All were autopsied cases ; the many liver cancers and cirrhosis cases diagnosed by biopsy were excluded. Classification was based on the microscopical sections ; in the majority of cases several slides were available and in many cases there were oversize sections, some up to lantern plate size, which made examination easy and accurate.

Cirrhosis in Kampala

Gross pathology

The cirrhosis at Mulago Hospital has been previously described (Davies, 1952) and a repetition is here unnecessary except in so far as it sheds information on the the cancer problem and is essential to this report. The livers were usually small, firm, tawny and nodular. Fibrous adhesions bound the organ to adjacent structures

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EXPLANATION OF PLATES.

FIG. 1.—Necroses and inflammatory cell change in post-necrotic cirrhosis : continuing activity. P.M. 170/54. × 50.

FIG. 2.—Laennec cirrhosis, Type A, to show connective tissue septa. P.M. 174/53. × 17.

FIG. 3.—Laennec cirrhosis Type A with central congestion. P.M. 354/56. Silver stain. ×50.

FIG. 4.—Laennec cirrhosis Type C. Showing large droplet fat in lesions of Laennec Type A cirrhosis. P.M. 25/54. × 50.

FIG. 5.—Laennec cirrhosis Type D, mild case. P.M. 159/52. × 50.

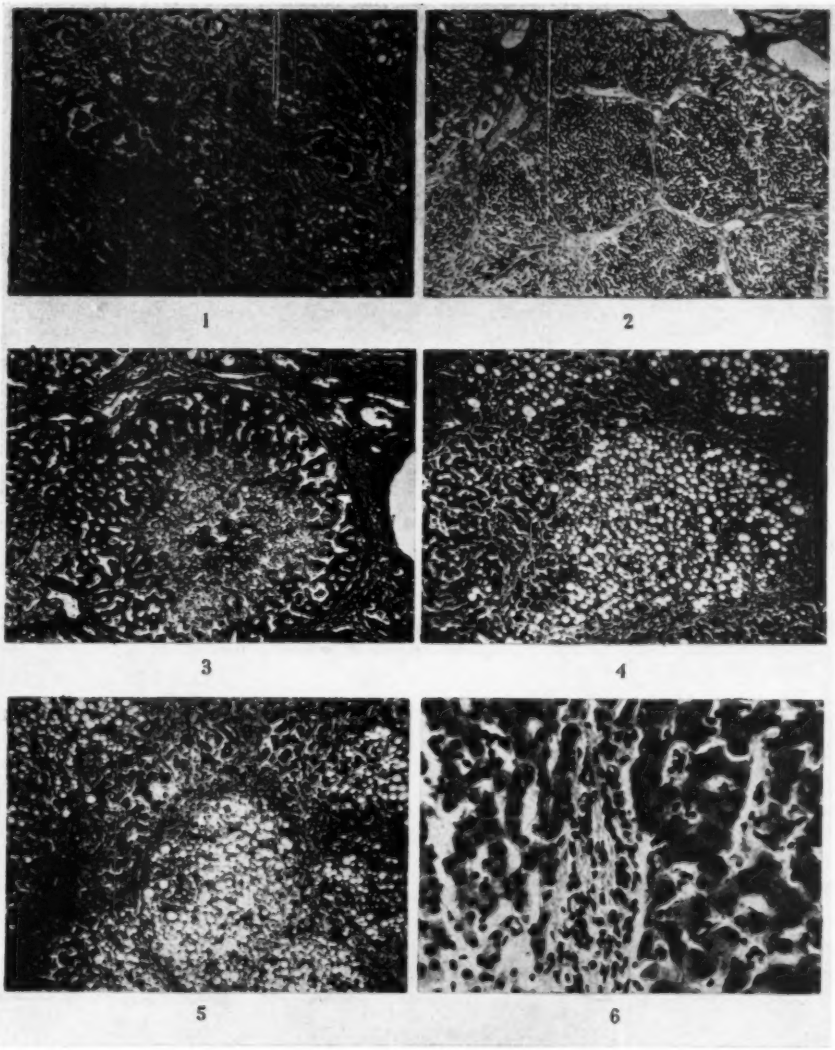
FIG. 6.—Liver cell atypia, in a case having a hepatocellular carcinoma. P.M. 299/55. × 210.

FIG. 7.—Laennec cirrhosis Type B with massive congestion. P.M. 10/53. × 24.

FIG. 8.—Hepacellular carcinoma Grade 4, having large cells. P.M. 91/51. × 290.

FIG. 9.—Cholangiofibrosis in a liver having both cancer and cirrhosis. P.M. 242/51. × 290.

FIG. 10.—Severe acute hepatitis, viral type. P.M. 152/53. × 65.



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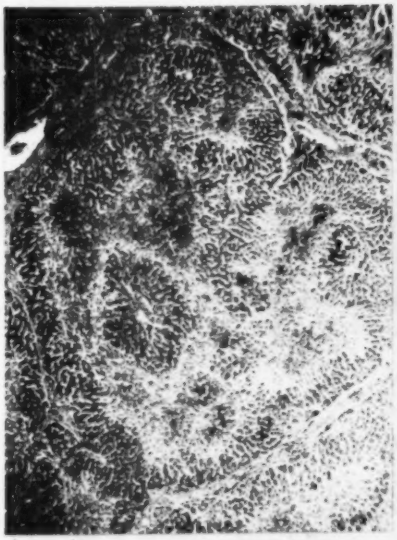

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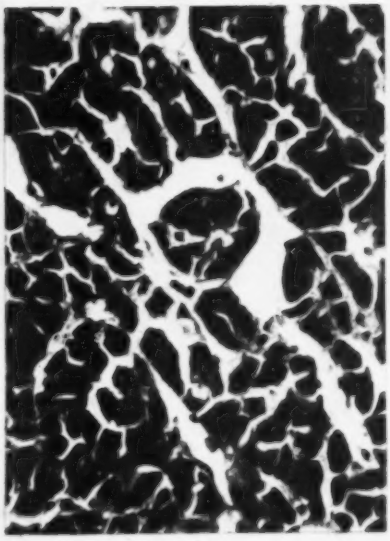

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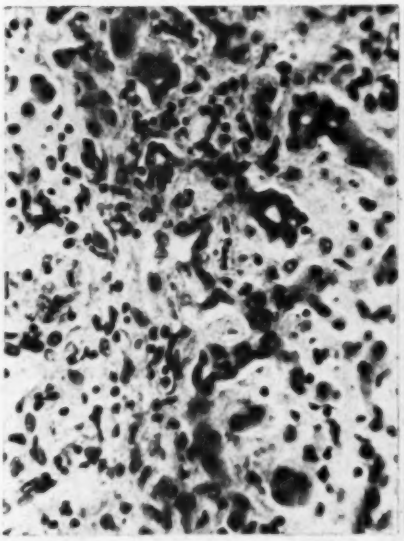

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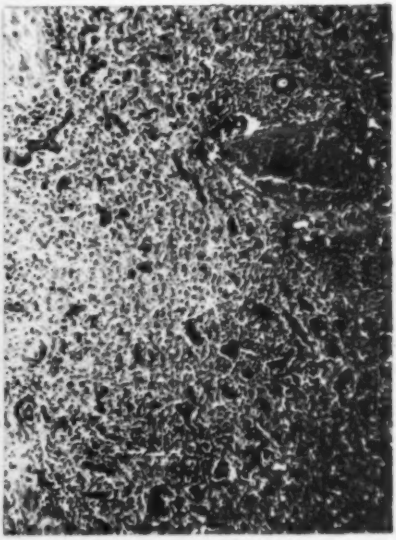

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in many instances. The spleen was usually enlarged, often very greatly so, and firm, showing the microscopic features of chronic passive congestion. It was often adherent to the surrounding structures by massive adhesions in which were very large vascular channels. The portal system was enlarged and the collateral circulation was developed. Death was however usually not due to haemorrhage from varices, perhaps because the dense adhesions provide safety valves, but to liver failure and coma or to intercurrent infection.

The liver was usually small; never was it greatly enlarged. The average weight in 40 cases without cancer was only 1237 g., the minimum was 565 g. and the maximum was 2675 g. The body weight at autopsy averaged 49·1 kg. (108 lb.). The nodularity was variable in size but was usually coarse; 15 mm. was a commonly recorded measurement for the larger nodules. The amount of scarring and its distribution likewise was variable but was usually severe. On the cut surface it was recorded a number of times as comprising about half the area. Grossly many livers were described as typical hobnail and diagnosed as Laennec cirrhosis. In others the scarring and nodularity were more severe, or affected one lobe more than the other, and the diagnosis was that of post-necrotic scarring. In the more typical cases of each the diagnosis was easy. Often however, the gross pictures merged and the diagnosis was a " toss-up ".

The liver weight averaged the same in the post-necrotic cirrhosis (1248 g.) as in the Laennec type B (1253 g.) to be described later. The other groups were not large enough to warrant comparisons but the livers seemed to be within the same range, except in the Laennec type A class where they averaged rather heavier.

Histopathology

A histological classification of the types of cirrhosis is given in Table I. It is clear that the great majority of cases are of either the post-necrotic type or the Laennec type, the latter predominating. Few other types were represented; one was a pigmentary cirrhosis (haemochromatosis) and two were of uncertain or mixed types.

The basis for this classification will be elaborated elsewhere. For purposes of this report it is sufficient to state that post-necrotic cirrhosis is that form which shows fibrous scars and bands, which are usually broad and which show evidence of collapse and condensation of liver stroma in the form of groups of blood vessels and bile ducts within the larger scars. The surviving liver tissue shows greater or lesser degrees of regenerative hyperplasia. The scarring and regeneration result in a distortion of the liver architecture. There is almost always evidence of the continuing activity of the process to be found at one place or another in the form of liver cell necrosis and acute to chronic inflammatory reaction involving either original liver tissue or regenerated nodules (Fig. 1).

The Laennec type A is the simplest chronic cirrhosis. It has bands or septa of connective tissue which subdivide the organ into nodules of various sizes which show also various amounts of regenerative hyperplasia, the whole resulting in a distorted liver architecture (Fig. 2, 3). The Laennec type B resembles type A but in addition shows small groups of blood vessels and bile ducts here and there within the fibrous septa as evidence of probable previous collapse of liver lobules and condensation of the stroma residues. Laennec type C resembles A or B but it has in addition much large-droplet fat in liver cells (Fig. 4). Laennec type D

resembles C but it exhibits in addition some fibrous tissue, usually more or less diffuse, within the liver nodules and connecting with the heavier and older septa that enclose the nodules (Fig. 5).

TABLE I.—*Histological Classification of Cirrhosis in Kampala Africans*

Type	All livers	Livers with cancer	Livers with no cancer
Post necrotic	38	13	25
Laennec Type A	13	11	2
B	43	26	17
C	0	0	0
D	3	0	3
Pigmentary	1	0	1
Mixed or uncertain	2	0	2
Totals	100	50	50

The one example of pigmentary cirrhosis resembled a Laennec type A except for the presence of large amounts of iron pigment in liver cells, Kupffer cells, bile duct epithelium, and in other elements of the scarred portal regions. Similar pigment was found in the pancreas, adrenal cortex and other organs. Central cirrhosis was not found in the fatal cirrhosis and only once in the non-fatal series. Biliary cirrhosis is rare in Africans and was only found in the non-fatal series.

It should be emphasised that in this classification some examples of Laennec type B and post-necrotic cirrhosis appear similar; both have areas of collapse, the post-necrotic cirrhosis having larger and more of such areas than the Laennec B type, so that the distinction becomes an arbitrary one at times. Also it was a common experience that some diagnoses of Laennec type A were later changed to type B as the examination of more and more slides finally revealed areas of collapse as already defined. Thus necrosis of liver cells, past and present, is an extremely common feature of cirrhosis in Kampala, occurring perhaps at many stages in a liver in which cirrhosis is already present. For these reasons it seems possible that these three kinds of cirrhosis, namely, post-necrotic and Laennec types A and B, are in reality only different degrees, stages and developmental forms of essentially the same basic process, and that they are identical in aetiology.

The observed facts are that most cases of cirrhosis at Mulago Hospital fall into three types which are closely related morphologically. The total picture was in fact remarkably uniform and clearly the possibility exists that there is one main aetiology for this cirrhosis. This is rather in contrast with experience in North America. This Kampala series was studied by one of us (P. E. S.) soon after reviewing a series of approximately 400 cases (200 fatal, 200 non-fatal) in Chicago. In each study a detailed work-sheet was made up to cover the histological details. This procedure was followed as an attempt to introduce as much objectivity into the study as possible and to quantitate the changes.

Comparison with American cases

A comparison of these records reveals some striking differences. Certain types of cirrhosis seen in Chicago, as for example the large fatty liver with cirrhosis, were absent in Kampala. In Uganda the cirrhosis picture is far simpler and the types

are fewer, but the cirrhosis appears to be more active. This is true of both the Laennec and the post-necrotic types. Evidence of inflammatory reaction and cell necrosis are much commoner in Kampala, as are atypical liver cells of the oversized hyperchromatic and multinucleated types. An intense inflammatory reaction is often found at the junction of the old fibrous septa and the liver tissue.

At the same time there are other important changes which in the African livers in Kampala are less conspicuous than in Chicago. Thus in Kampala regeneration of liver cells is less conspicuous and the ratio of estimated new to old (original) liver cells is far lower on the average in Kampala than in Chicago. In many cases nodules of liver separated from each other by fibrous septa are composed of one or more anatomical lobules which appear to be composed of original liver cells. Such nodules are seen both in the post-necrotic and the Laennec cirrhosis. Thus not only is there less nodular regeneration in Kampala but more of the nodules appear to contain many anatomical liver lobules rather than one, and in Kampala, therefore, most of the cirrhosis is multilobular rather than monolobular. Bile duct proliferation is also less conspicuous in the African livers and far fewer African livers have appreciable increases in fat. The intense fatty liver of kwashiorkor occurs in a much younger age group than we are here concerned with and adult cases are rarely seen now in Kampala. The consequence of all these differences from the Chicago pattern is that the picture of cirrhosis in this part of Africa is far more uniform. Incidentally no histological differences could be noted between the cirrhosis of the White and of the Negro in Chicago.

In Kampala practically all the cirrhosis appeared to be portal although because of its multinodular nature some portal regions were spared in many cases ; however, they usually showed a mild degree of stellate fibrosis. The central areas are often not involved. This can best be demonstrated in livers in which a terminal acute passive congestion serves to delineate the centre of the lobules (Fig. 7).

Aetiological factors in cirrhosis in Kampala

Race.—A total of 23 African tribes is represented by these 100 cases. Nearly all are Bantu but a few were of Nilotic or Hamitic types. The tribal representation was as follows : Rundi 43, Ganda 10, Nyoro 6, Ankole 4, Acholi, Teso and Lugwala 3 each ; Kiga, Gishu, Muzinja and Ziba 2 each ; twelve tribes, ranging from the Congo to far into Kenya and from the Sudan to Tanganyika had one each. The large number of Rundi is notable. They are immigrants to Uganda from Rwanda-Urundi. Their prominence in this series may represent no more than the fact that they are immigrants and thus if incapacitated in hospital have no home to return to and no relatives to claim the body if deceased. On the other hand they may genuinely be more liable to develop cirrhosis.

Sex.—Eighty-nine of the cirrhoses were males and eleven were females. A similar male preponderance was previously reported (Davies, 1952) and the point was made that this ratio is almost the same as that of the total autopsy population of the two sexes. It cannot thus be said that cirrhosis is more common in the male African, though in most places cirrhosis is commoner in the male. The significance of this remains unknown.

Age.—The average age of 93 cases with cirrhosis was 35 years. The youngest was 12, the oldest 65 years. This youthfulness of the cirrhotic group, as will also be seen in the cancer group, is the result of the age structure of the population

and does not represent a greater risk of attack in the young than in the older person. Nevertheless the occurrence of so many cases at a young age does show the presence of factors capable of damaging the liver severely. In the Chicago series the average age in 180 cases of fatal cirrhosis after excluding the congenital obstructive cirrhosis was 53 years.

Infection.—As previously indicated many livers with either post-necrotic or Laennec cirrhosis showed much inflammatory reaction (Fig. 1, 4, 5). This was often so great as to be designated a hepatitis. This hepatitis was variously acute, subacute or chronic and usually regional rather than diffuse. It was sometimes related to liver cell damage (Fig. 1), but often it appeared topographically unrelated to injured cells and it was often too excessive to be regarded solely as a reaction to necrotic liver cells. The nature of the hepatitis producing factor is not known. It may be viral. Infectious hepatitis is sometimes diagnosed in Uganda African adults but little is known as to its frequency. Some cirrhotics give a history of a previous attack of jaundice but this is inconstant and it is not clear how often this is due to a failure of memory or to a failure of communication. Many Europeans in Uganda at some time or another have an attack of infectious hepatitis. A few cases of hepatitis come to autopsy and many cirrhotic livers show areas of active inflammation, that could be regarded as due to a viral hepatitis (Fig. 1). Cases of acute necrosis are not very uncommon, but acute haemorrhagic hepatic necrosis is very rarely seen. The evidence would thus seem to point to a viral hepatitis as a probable cause of most of the cirrhosis. Probably this is the virus of infectious hepatitis but proof depends on demonstration of the virus. A test for infectious hepatitis is very badly needed in tropical Africa.

In most countries cirrhosis is believed to result infrequently from infectious hepatitis, although the frequency of this sequel may be under estimated. If, in Uganda, a high frequency of this relationship can be demonstrated it will have to be explained. It has been stated that homologous serum hepatitis is more severe in the malnourished (Wood, 1946) and it may be that malnutrition in Uganda plays an important secondary part in producing cirrhosis.

Miscellaneous factors

Schistosomal infection is not an important cause of cirrhosis in Kampala. It was only seen in three livers in this series, one each in the fatal cirrhosis, the non-fatal cirrhosis and the non-cirrhotic groups. In the latter case there were a few well encapsulated miliary granulomas but no cirrhosis. Another liver showed a mild chronic pericholangitis and an occasional interportal fibrous septum associated with schistosomal granulomas in the ileum. Most of the cirrhotic livers had little or no fatty change. In only a few cases was fat conspicuous, and in some of these there was tuberculosis elsewhere in the body. In the non-fatal cirrhosis group in which the lesion was either mild or early, there was no evidence that the cirrhosis evolved from a fatty liver. In the miscellaneous group were several cases of kwashiorkor with severe fatty livers. They had some portal scarring but no cirrhosis. In this area the evidence points to a cirrhosis-producing agent accompanied by little or no fatty change. Similarly the principal cause of cirrhosis in Uganda is not related to any significant disturbance in pigment metabolism. Only one case of pigmentary cirrhosis was seen and one case of haemosiderosis without cirrhosis was studied.

Carcinoma of the Liver

Seventy cases were studied.

Gross pathology

Many, and probably all gross types of primary liver cancer were represented among these cases, including the massive, nodular and diffuse. The appearances were like those described elsewhere (Berman, 1951 ; Ninard, 1950 ; Davies, 1952) and a repetition of the details is not necessary. No unusual features were observed in Uganda except that the tumours were very large on the average. The enlargement was due to the tumour and sometimes there was very little uninvolved liver tissue remaining. The average weight in 44 cases was 3045 g., the smallest was 1320 g. and the largest was 7100 g. Considering that most of these people are normally small and that the average body weight was only 46·4 kg. (102 lb.) this represents a great enlargement. It was greater than the averages reported (2547 g. and 2610 g.) in the two series from the U.S.A. where the average body weight is nearly fifty per cent more. It is probable that the large size of the tumours together with their friability explains the common method of death by rupture of the cancer through the liver capsule with a fatal intraperitoneal haemorrhage which occurs in about one-fifth of the cases.

The weight of the liver and the tumour was considerably greater in those cases that had no cirrhosis (average 4134 g. in 9 cases) than in those with cirrhosis (average 2768 g. in 35 cases). This is interpreted as showing that the co-existence of cirrhosis with cancer hastens the onset of failure or of some other complications or sequelae so that the tumour does not have the opportunity to grow so large. The tumours frequently were disseminated through the liver, and the portal and hepatic veins often had tumour thrombi in them. Despite the frequency of this intravascular growth, even more conspicuous microscopically, and the large amount of tumour present, distant metastases were recorded in less than half the cases, a figure similar to that recorded from other regions. The paucity of metastases may be due to the sheathing of the tumorous cords by endothelial-like cells. The commonest sites for metastatic growths were the portal lymph nodes and the lungs. The localisations in other organs were not perceptibly different from those reported in the literature. Death by haemorrhage from varices was very uncommon.

A primary carcinoma of the liver in Uganda is usually readily recognised by its gross appearance (Davies, 1952). Less commonly it resembles a metastatic cancer because of multiple growths of approximately equal size, and rarely it is hardly discernible when the tumour nodules are small and discoloured in a highly cirrhotic jaundiced organ. Usually the primary growth is large ; it may become massive by confluence. Basically it is yellowish-red (liverish) or off-white in colour. The advanced stages tend to appear multinodular owing to intrahepatic metastasis, to large tumour thrombi in veins, or to the substitution by tumour of the hyper-plastic nodules of hepatic tissue in a cirrhotic liver. Those tumour nodules located beneath the liver capsule are usually not umbilicated. It is usually possible to distinguish the less common cholangiocellular carcinoma from the hepatocellular type in the gross by its paler greyish-white or pinkish-white colour, firmer consistency, and fewer secondary colour and degenerative changes (Davies, 1952).

Histopathology

One of the tumours was a cholangiocellular carcinoma and the other 69 were hepatocellular carcinoma. The latter usually showed cellular cords variable in size in either a sinusoidal or trabecular form. The cells tended to retain some resemblance to hepatic cells in their arrangement and in cytoplasmic and nuclear appearances. In many cases lumina were seen within some of the cords but this feature did not alter the diagnoses of hepatocellular carcinoma, such a type of growth being regarded as pseudo-cholangiomatous, the cells lining the lumens retaining their ability to secrete bile, their eosinophilic cytoplasm and their nuclear and nucleolar resemblance to liver cord cells.

No marked differences were seen in these carcinomas from the two series studied in the U.S.A. except for a higher proportion of the hepatocellular type (Edmondson and Steiner, 1954) and a greater proportion showing much cell anaplasia. When the hepatocellular carcinomas were graded on the basis of their morphological deviation from normal liver cells (Edmondson and Steiner, 1954) 3 were in Grade II, 26 were in Grade III and 36 were in Grade IV. The degree of anaplasia is thus very high. Four were not assigned to any grade as there was variation of appearance from one area to another.

A small proportion showed evidence of function in the form of bile formation, glycogen storage and fat accumulation. The metastases were often bile stained. The distribution and proportion of silver positive fibrillar material was no different from that seen in other geographical areas. The tumour metastases in the liver and elsewhere usually resembled the primary growth and their appearance and behaviour were similar to those seen elsewhere.

Aetiological factors

Race.—All were Africans, mostly Bantu, and a few were Nilotes and Hamites, sixteen different tribes being represented. Chief of these were the Rwanda-Urundi with 23 cases, Ganda with 11, Nyoro 9, Acholi 4 and Toro. 3 The Kiga, Gishu, Lugwala, Ankole and Madi people were each represented by two cases, the Muvuma, Muzinja, Ziba, Bori, Kikuyu and Nubi by one each, while in 4 cases the tribe was not stated.

No special aetiological deduction can be drawn from this tribal distribution at this time. The same array of tribes is found in other diseases. The Rwanda-Urundi predominate, being often homeless immigrants. The course of disease being so rapid it cannot be stated if liver cancer is frequent in the true homes of these tribesmen. It is possible that the incidence of liver cancer is really higher in the Rwanda-Urundi and a survey now projected in their homeland should provide an answer in due course.

Sex.—Sixty-two were males and only 8 were females. This cancer is known to be commoner in males in all areas and these figures are probably correct for Uganda being in consonance with the incidence figures previously stated (Knowelden, 1957), where no excess in liver cancer in females in Uganda was found. In previous reports (Davies, 1952, 1954) some observations were recorded suggesting that hepatocellular carcinoma arose more frequently in females in the absence of cirrhosis, than it does in males.

Age.—The average age of 67 persons in whom the age was recorded was

36 years ; the youngest was 12 and the oldest was 70 years old. The age distribution was as follows :—

Years	Number of cases
10–19	2
20–29	11
30–39	32
40–49	14
50–59	7
60–69	0
70–79	1
Unstated	3

The amazingly high number of cases in the early decades of life is shown by these figures. The aetiological factors appear strong regardless of whether they are intrinsic or extrinsic, environmental or inherent.

These dates represent the case age distribution and do not show the incident rate or measure risk to the disease and it reflects the youthful age structure of the population. A previous statistical analysis of the liver cancers showed that the proportion in autopsies does not vary much with age (Davies, 1952).

The average age in 40 cases of liver cancer in Chicago was 58·3 years and in the large Los Angeles series it was 58·6 years. Thus the average in the Uganda cases is about two decades younger. In view of the greater " activity " of the cirrhosis in Uganda, as judged by the amount of inflammatory infiltrate, and that abnormalities of the liver cells in the non-tumerous parts of the liver are more conspicuous, it seems that we have evidence of severe liver damage, and if the incidence rate eventually proves to be exceptionally high, as now seems probable, then it is probable that the disease often comes on early in life due to a shorter induction time.

Cirrhosis with carcinoma

Fifteen carcinomas (21 per cent) occurred in livers having no cirrhosis and 55 (79 per cent) were in cirrhotics. This proportion is within the range recorded elsewhere.

The types of cirrhosis in the 55 cases was classified as follows :—

Type	Number of cases
Post necrotic	12
Laennec, untyped	2
Type A	12
B	25
C	0
D	1
Florid and unclassified	3

Miscellaneous factors

None of the 70 cancerous livers showed evidence of schistosomiasis or other recognised parasites. None had a noticeable excess of iron pigment in cells of any type. Evidence of vascular thrombosis was sought but apart from the tumour thrombi none was found. It has been emphasised that fat was not a conspicuous feature in these livers.

Cytopathogenesis

The non-neoplastic cell or cells which immediately precede the cancer were not identified in this study and neither were any definite intermediates. Abnormal

epithelial cells were seen in the livers both with and without cirrhosis but it is highly questionable whether they are in the direct line of cytogenesis because the cancers which eventually appear do not resemble them. Thus in the non-cirrhotic liver bearing also a cancer, groups of atypical, enlarged, often multinucleated liver cells are commonly found. Their disorderly arrangement and the enlarged hyperchromatic nuclei with conspicuous nucleoli suggest that they might be incipient cancers (Fig. 6). These groups of cells sometimes give an impression of growth by their compression of the surrounding liver cells. They are the most obvious abnormality of epithelial cells in the non-cirrhotic cancer livers, yet the cancers arising in these livers appear quite different from them. In the present series of 70 cases only one bears even a remote resemblance to such cells (Fig. 8). It would thus appear that they can hardly be the precursor of the generality of liver cancers.

In the livers with cirrhosis, a greater array of abnormalities of epithelial cells is seen. Beside (*a*) the atypical enlarged cells already mentioned in the non-cirrhotic liver, may be mentioned the following: (*b*) proliferating bile ducts (Fig. 9), (*c*) proliferated cholangioles and the related condition of cholangiofibrosis; (*d*) atrophic cells with small hyperchromatic nuclei; (*e*) mixtures of the above. Yet apart from an occasional cholangiocellular carcinoma (only one in this series), the liver cell cancers do not resemble these cells which one is tempted to designate pre-cancerous. Moreover in Uganda the epithelial hyperplasia appears quantitatively less than in Chicago, true regeneration nodules are less frequent, yet cancers are common.

In several cases of fatal acute hepatitis without cirrhosis, probably viral in type, highly abnormal forms of liver cells were seen which also suggested pre-cancerous lesions (Fig. 10). Some cells were enlarged, others were reduced, the nuclei were abnormal and the general impression again was one of young proliferating cells. That these forms are not the usual precursor lesions of cancer is probable from the facts that carcinomas do not have cell mixtures of that sort; cancer does not follow hepatitis which is in that acute stage, and when it supervenes in a liver which previously had a virus hepatitis these cell forms are absent.

DISCUSSION

In this study we have sought only to compare and contrast the histopathology of liver cirrhosis and cancer in Africans in Kampala with the same diseases as they occur in both the Negro and White people of North America. Both similarities and differences emerge from this comparison. Firstly hepatocellular carcinoma is the major, indeed virtually the only problem in Uganda, occurring in young people, with an average age of thirty and preponderantly in males. Very large livers are produced both with respect to absolute weight and relative to body weight, the cancerous livers without cirrhosis being larger than those with cirrhosis. There is a wide range of gross morphological types, but neither grossly nor histologically do these differ from types seen in America. There is nothing new or exotic about liver carcinoma in Uganda as regards morphology. We differentiate sharply between the cholangiocellular carcinoma and the hepatocellular, as does Higginson (1956) in Johannesburg and Shanmugaratnam (1956) in Singapore. The cholangiocellular form is uncommon in Africans and probably is seen relatively less frequently than in Europeans or Americans. The majority of cases of hepatocellular carcinoma are associated with cirrhosis, 79 per cent in this series, but

there is a substantial proportion arising in both countries in non-cirrhotic livers. The cirrhosis in Uganda is of an active type, there is evidence of past liver cell necrosis and collapse and such a degree of inflammatory reaction is often present as to warrant its being designated as post-hepatitic and the probable cause is a viral one. The cirrhoses can be classified, with some overlap, as post-necrotic and Laennec cirrhosis, the latter of the form in which there is little or no evidence of post-necrotic collapse foci. The cirrhotic picture, thanks to the frequency of post-necrotic lesions was therefore remarkably uniform. Although the fibrosis was often dense and severe, and of considerable standing, the livers also tended to show an active process either superimposed or in continuation in the form of the aforementioned inflammatory reaction, liver cell necrosis and the presence of atypical large liver cells. On the other hand the following features tended to be relatively inconspicuous as judged by the cirrhosis in some other countries; regeneration of liver cells with nodular hyperplasia and the ratio of new to old liver cells, fatty change, and bile duct and cholangiole hyperplasia. Finally the cytopathogenesis of the liver cancer is not clear.

We can therefore provide partial answers to the questions asked previously—thus (1) the high incidence is due to more cases of the usual morphological types of cancer with no new varieties; (2) the cirrhosis accompanying liver cancer in Uganda is an active cirrhosis in which lesions occur suggestive of a causative hepatitis, possibly viral in aetiology; (3) there are a variety of changes in the African liver which might be concerned with carcinogenesis but there is difficulty in accepting any of them as pre-cancerous lesions, and (4) the aetiology of the cirrhosis can be explained granting certain assumptions but the aetiology of the carcinoma remains quite obscure.

Considerable discussion could be devoted to these findings but would be profitless in view of the many obscurities which surround this field. Much work is going on in Africa on these problems and further detailed information is to be hoped for, and if some uniformity of nomenclature can be introduced this will be very advantageous. Some of the confusions have arisen through differences of nomenclature, others through differences of objective. Some have been concerned to show that carcinoma is very frequently a complication of cirrhosis, others to show that it arises sometimes in non-cirrhotic livers. A synoptic view must be taken while at the same time emphasis on the differences may lead to advances as in other fields of scientific research.

One of us has in a series of papers (Davies, 1952, 1954) pointed out some of the differences and distinctions which may in Africa be of help in elucidating the aetiology. It is interesting that the cirrhosis was classified into post-necrotic scarring and into Laennec cirrhosis in those papers on rather different grounds but with almost identical results. The point was there made that liver carcinoma arose most frequently in livers showing an old Laennec cirrhosis rather than in livers with acute post-necrotic scarring. It was therefore argued that this threw some doubt on the postulated relationship. Higginson (1956) has recently criticised this argument and suggested that in S. Africa the carcinoma arises out of the hyperplastic nodule, as is recorded in animals (Firminger, 1955). Yet by an entirely different approach to that previously adopted, namely by a direct contact between Uganda and American livers we have concluded that the evidence for regeneration and hyperplasia is less in the Uganda livers than in the American. Moreover we have been unable to satisfy ourselves that any of the types of cellular

activity seen in these damaged livers are precursors of malignant change. It is also of interest that we, like other workers in the tropics, have not found cases of mixed cholangio-hepatocellular carcinomas such as are frequently found in experimental liver cancer in animals, and in the places having liver flukes.

We are still left with the problem of the relationship of cirrhosis to carcinoma. It is obvious that a large number of carcinomas arise in a cirrhotic liver, it is equally obvious that carcinoma arises not infrequently in a non-cirrhotic liver. Our evidence is against the view that carcinoma arises out of the hyperplastic nodules, for these are less common in African cirrhosis and less common still, though this can occur, in non-cirrhotic livers. Perhaps the cirrhosis is no more than an indicator of severe liver damage without being in itself a direct precursor lesion of carcinoma.

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PROGRESSION OF LIVER LESIONS PRODUCED IN RATS BY TEMPORARY TREATMENT WITH PYRROLIZIDINE (*SENECIO*) ALKALOIDS, AND THE EFFECTS OF BETAINE AND HIGH CASEIN DIET

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WE have shown previously that various pathological liver changes, including malignant hepatoma with metastases can be produced in rats by intermittent administration of pyrrolizidine alkaloids throughout the life of the animals (Cook, Duffy and Schoental, 1950; Schoental, Head and Peacock, 1954; Schoental and Head, 1955).

In view of the difficulty of assessing the degree of malignancy from microscopic examination of liver lesions, in the absence of metastases, it seemed important to obtain some other criteria which could throw light on their nature, such as their progression or regression when the treatment is discontinued.

We now report the results of experiments in which essentially similar liver lesions, including hepatoma, developed in rats which were treated for only restricted periods with the alkaloids and then were left without any further treatment till death. The alkaloids used were: retrorsine and its N-oxide isatidine, tested previously, and riddelliine, another pyrrolizidine alkaloid from *Senecio riddellii*, not so far tested in rats.

In addition the effects of supplements of betaine and of high casein diet on the development of the liver lesions were explored.

MATERIALS AND METHODS

Young Wistar rats, bred locally, were used. In the same way as in the previous experiments of this series the animals were housed in metal cages, 2 to 6 per cage, segregated by sex, and were given food and water, or the appropriate solutions, *ad libitum*. Their "normal" diet consisted of commercial pellets, Shearer's Pig Weaner Nuts No. 1 (for its composition see Schoental *et al.*, 1954) replaced in later stages of the experiment by M.R.C. Diet 41 (Bruce and Parkes, 1949). When high casein diet was used this was given in the form of a powder obtained by mixing:

	Per cent
Unextracted casein (Glaxo) . .	75·0
Ground maize (Shearer) . . .	12·0
Dried yeast (Boots)	2·0
Salt mixture (Glaxo D.L.6) . .	4·5
Arachis oil (T. & H. Smith) . .	5·0
Cod liver oil (T. & H. Smith) . .	1·5
	100·0

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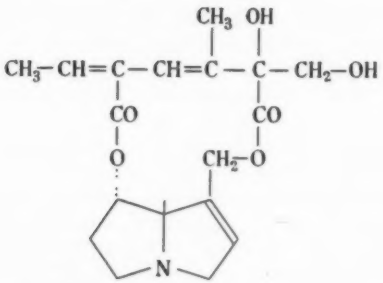
When " normal " diet was supplemented with betaine the latter was given as a 1 per cent solution in tap-water, neutralised with dilute hydrochloric acid.

The alkaloids, retrorsine and isatidine, were the same crystalline compounds as used in previous experiments. Crystalline riddelliine was a gift from Professor Roger Adams who established its structure (Adams, Hamlin, Jelinek and Phillips, 1942). It is a cyclic ester of retronecine with riddelliic acid (Fig. 1).

For injection, the solutions of the alkaloids were freshly prepared in water and neutralised with dilute hydrochloric acid. For feeding purposes stock solutions similarly prepared were stored in the cold (about 5° C.) for short periods, and diluted when required.

All the experimental and control rats were weighed at approximately weekly intervals. In some rats liver biopsies were performed under ether anaesthesia.



Riddelliine

FIG. 1.—Chemical formula for riddelliine.

A part of the presenting lobe was then removed for microscopic examination. The animals that died or were killed by chloroform when moribund, were examined *post mortem*. The livers and some other organs were then fixed in " formal corrosive" for microscopic examination ; sections were routinely stained with haematoxylin and eosin ; other fixatives and stains were used when required for special purposes.

EXPERIMENTAL

In Table I are summarised the treatments and survival times of all the animals.

Riddelliine

Fourteen female and 6 male rats on " normal " diet were given solutions containing 0·02 mg./ml. of riddelliine in drinking water twice weekly during 6 months. Five of the females and 5 surviving males were then injected intraperitoneally with riddelliine, 25 mg./kg. body weight ; 3 such injections were given in the course of one month. The remaining female rats continued to receive solutions of the alkaloid. One year after the beginning of the experiment all the surviving rats, 12 females and 4 males, were each injected intraperitoneally with riddelliine 30 mg./kg. body weight and were left without further treatment till death.

TABLE I.—*Survival of Rats Treated with Pyrrolizidine Alkaloids*

Alkaloid	Diet	No. of rats	Sex	Dosage	Route	No. of rats which died: Less than 1 year	1–1½ years	1½–2½ years
Riddelliine	N	14*	F.	0·02 mg./ml.+inj. 25–30 mg./kg.	O.+I.P.	2	4	8
	„	6*	M.	Ditto	„	2	3	1
Retrorsine	N	2	F.	6×25–30 mg./kg.	I.P.	—	—	2
	B+N	2	„	„ „ „	„	—	—	2
Isatidine	N	3	M.	0·2 mg./ml.	O.	3	—	—
	B+N	3	„	„ „	„	3	—	—
	„	7*	„	0·03 mg./ml.	„	4	2	1
	N	6	„	5×40–50 mg./kg.	I.P.	3	2	1
	C+N	6	„	„ „ „	„	2	1	3
	N	6	F.	„ „ „	„	3	3	—
	„	3	„	5×25–50 mg./kg.	„	—	—	3
	„	5	M.	„ „ „	„	1	2	2
Controls	„	8	„	—	—	—	—	8
	„	8	F.	—	—	—	—	8
	B+N	3	M.	—	—	—	—	3
All		82	M. and F.					

N = "Normal" diet; B = betaine supplements; C = high casein diet; * = biopsies performed; F. = females; M. = males; O. = oral; I.P. = intraperitoneal injections.

Liver biopsies were carried out on 2 of the male rats 2 months after treatment was started and on microscopical examination anisocytosis was seen in both livers. This was more pronounced in one which showed areas of small basophil cells containing much glycogen alternating with areas of large eosinophil cells containing less glycogen. Many of these cells had large nuclei and eosinophil inclusion bodies were seen in the cytoplasm, but no fatty change was noted.

Little change was seen apart from congestion and dilatation of sinusoids in biopsies after 3½ months and at 6 months some thrombosed blood vessels and fatty change were noted.

One male rat was killed 4 months, and one died 11 months after treatment started. In the liver of the former small pale microscopical nodules of vacuolated cells were seen adjacent to portal or hepatic veins and some vessels were thrombosed, and in the latter areas of degeneration and regeneration were seen in the liver.

Of the 4 males which survived after one year of treatment, 1 died and 2 were killed 6 months, and one 16 months after the last injection. The livers of all 4 rats were grossly abnormal, showing pale solid nodules and in 2 cystic areas were also present. In the rat which died after 6 months the liver weighed 30 g. and nodules were present in all the lobes, the largest (3 × 2·7 cm.) being in the left half of the median lobe. Blood was present in the abdominal cavity. On microscopical examination the liver showed nodular hyperplasia and fibrosis with areas of bile duct proliferation (Fig. 2). Many nodules had necrotic centres and areas of haemorrhage.

The 2 male rats which were killed after 6 months had pale nodules in the liver but fibrosis was not seen. Microscopically some of the nodules were composed

of large vacuolated cells with faintly staining eosinophil cytoplasm. Binucleated cells were common and some mitoses were noted. Some of the cells contained phloxinophil inclusion bodies. These nodules usually had one side adjacent to hepatic or portal veins. Other nodules were composed of foamy, fatty cells. In one, a larger nodule composed of basophil cells with a trabecular arrangement may be an early hepatoma (Fig. 3).

The fourth rat which was killed 16 months after the last injection had a nodular liver, some nodules being pale and solid and others being cystic. The spleen and kidneys were congested. Microscopically the nodules were similar to those seen in the earlier cases but were larger and mitoses were more common. Much bile duct hyperplasia was present round the portal systems and extending between the liver cords. Multilocular bile duct cystadenomata were present. Pigmented histiocyte-like cells were present in the hepatic veins similar to those seen in rats treated with retrorsine and isatidine in previous experiments (Schoental, *et al.*, 1954). The kidneys and spleen in all 4 rats were congested and much pigment was seen in the spleens. All the lungs showed areas of bronchiectasis.

Two female rats were killed 11 and 12 months after the start of treatment. The former showed fatty change in the liver. A biopsy at 3½ months had shown only congestion. The latter showed microscopical nodules of small regenerating cells and of large faintly staining granular cells. Areas of fatty change were also noted. The spleen contained much stainable iron pigment and the kidneys showed catarrhal change.

Twelve female rats died or were killed 3–17 months after the last injection of the alkaloid. The livers were less severely affected than those of the male rats which received similar treatment; 5 rats which died after 3–8 months showed only slight congestion of the liver. In the livers of 6 female rat which died after 10–17 months, multilocular bile duct cysts were present and 5 had also small yellow nodules. One rat had a sarcoma in the liver arising in the wall of a tapeworm cyst, with metastases in the abdominal lymph nodes. The liver also showed centrilobular necrosis with a zone of fatty change at the periphery of the lobules.

On microscopical examination the small yellow nodules in the liver were similar to those seen in the male rats. They consisted of small regenerating cells, of pale vacuolated granular cells and of foamy, fatty cells. The spleens contained much pigment and the kidneys were congested in most of the rats. Bronchiectasis was present in 9 animals, haemorrhage into the stomach in 2, a mammary fibroadenoma in 1, pyometra in 1 and a large abscess in the lower jaw in 1.

Retrorsine

Four female rats (100–120 g. body weight) were each given 6 injections of retrorsine (25–30 mg./kg. body weight); 4 of these were given approximately at weekly intervals during the first month and were followed by 2 injections given every 2 months. Two of the rats were given solutions containing 1 per cent betaine instead of drinking water during 1 year beginning 24 hours before the first injection of retrorsine. The latter 2 rats survived 28 and 29 months from the beginning of the experiment, or 22 and 23 months respectively after the last injection of the alkaloid. Both had grossly nodular livers, one of which contained a hepatoma (Fig. 6, 7). All 4 rats had large multilocular cysts in the livers (Fig. 4, 5).

The 2 female rats which received the same course of retrorsine injections but did not receive betaine had only microscopic nodules in their livers when they died 19 and 22 months respectively after the last injection of the alkaloid. The growth rates of both groups of rats were almost identical till shortly before death and not unlike those of female control rats, and the animals appeared to be in good health for at least 2 years after the beginning of treatment.

Isatidine

Feeding

(*a*) Six male rats (60–70 g. body weight) were given solutions of isatidine containing 0·2 mg./ml. instead of drinking water twice weekly during one month. Three of these rats received additionally daily solutions of betaine beginning 24 hours before alkaloid treatment.

The 3 rats given only solutions of isatidine and 1 from the betaine-treated group died less than 7 weeks after the beginning of the experiment. The livers of these rats showed mostly centrilobular necrosis. Histiocyte-like cells were seen in the hepatic veins and also in the small veins in the lungs which were congested. The kidneys and spleens were also congested. Gastrointestinal haemorrhage was present in the betaine-treated animal.

Two of the rats which received concurrently isatidine and betaine survived 3½ and 4 months respectively. These remained stunted, sexually immature, and developed striking anaemia and generalised oedema. *Post mortem* they were found to have pleural and peritoneal effusions of serous fluid, the lungs showed haemorrhagic petechiae, the livers were shrunken, granular, the pancreases oedematous, retroperitoneal lymph nodes were dark red, and the kidneys were dark brown. Microscopical examination of the livers showed areas of degeneration and regeneration. There was an increase in bile duct formation and some fibrosis. Stainable iron pigment was present in the portal triads. There was great variation in the size of the parenchyma cells. The features seen in these two rats were similar to these described previously (Schoental, 1955).

(*b*) Seven male rats (50–100 g. body weight) were given solutions of isatidine 0·03 mg./ml. twice weekly and 1 per cent solution of betaine daily for about 1 year.

Liver biopsy specimens were taken 1 and 3 months after the beginning of treatment. Two at 1 month showed some hyperplasia of liver cells which formed tortuous cords. Two at 3 months showed congestion of sinusoids with some thrombosis of the central veins in one. One of the biopsied rats (256/54) died after 3 months and showed necrotic tissue at the site of the previous biopsy, but otherwise the liver architecture was normal.

The next rat (734/54) died after 6½ months and had nodules in all lobes of the liver, some about 2–3 mm. diameter. The other organs were congested and large haemolymph nodes were seen in the omentum. Microscopically, numerous nodules of hyperplasia were seen. Some with areas of degeneration in the centre had a hepatoma-like appearance.

Rat 1171/54, killed after 10½ months had a large smooth dark liver and showed macroscopical iron reaction in the liver, spleen and lungs. On microscopical examination the liver cells were large and swollen and some showed fatty change. There was some thrombosis of blood vessels.

Rat 1348/54 died after 1 year and nodules 2–3 mm. diameter were present in all the lobes of the liver. Microscopically these were of two different types. One was composed of large granular, vacuolated cells in which mitoses were present and the other of small basophil cells. They were surrounded by flattened liver cells.

Rat 716/55, 6 months after treatment ceased, had numerous nodules in all lobes with almost no normal liver tissue. A large pale tumour-like mass was present on the under-surface of the median lobe. Microscopically these showed hyperplastic liver cells and some had hepatomatous appearance. Other areas showed cystic bile duct formation and fibrosis was present round the nodules.

Rat 809/55 was killed 3 weeks later and had a large liver with several nodules 0·5–1 cm. diameter and cystic areas. The other organs were congested. Microscopically the nodules were composed of hyperplastic cells some probably hepatomata. Only a little fibrosis was present.

One rat (1091/55) had a large tumour-like area in the liver and a haemorrhagic nodule in the omentum when it died 10 months after treatment stopped. The tissues were autolytic but nodules of irregular hyperplasia could be made out on section. The nodule in the omentum contained giant cells surrounding a central area of haemorrhage.

Intraperitoneal injections

(*a*) Twelve male and 6 female rats were given 5 injections of isatidine 40–50 mg./kg. body weight each in the course of the first 2 months of life, the first injection being given before weaning when the rats were 17 days old.

Six of the male rats received from weaning the high casein diet for 6 months, followed by " normal " diet till death. The other 6 males and 6 females were given " normal " diet all their lives.

The 6 rats on the high casein diet survived from 10 to 23 months after the last injection of the alkaloid.

EXPLANATION OF PLATES.

FIG. 2.—(525/55). Male rat 6 months after treatment with ridelliine had stopped. Nodular hyperplasia and fibrosis. H. & E. × 25.

FIG. 3.—(533/55). Male rat 6 months after treatment with riddelliine had stopped. Part of hepatoma-like nodule in upper right of field with flattened pernchyma cells in lower left corner. H. & E. × 175.

FIG. 4.—(587/56). Female rat 22 months after the last subcutaneous injection of retrorsine. Supplements of betaine were given during the first year. Large cysts are present in all lobes and some solid, pale nodules.

FIG. 5.—(587/56). Section of multilocular cyst shown in Fig. 4. H. & E. 25.

FIG. 6.—(706/56). Female rat 23 months after the last subcutaneous injection of retrorsine. Supplements of betaine were given during the first year. A large yellow tumour-like mass is present in the left lobe, small yellow nodules in the middle lobe, and cysts in the right lobe.

FIG. 7.—(706/56). Section of tumour shown in Fig. 6 showing variation in structure—partly solid and partly cystic. H. & E. × 22.

FIG. 8.—(812/55). Male rat 11 months after the last injection of isatidine. High casein diet was given for the first 6 months of life. Fatty nodule. H. & E. × 135.

FIG. 9.—(744/56). Male rat 23 months after last injection of isatidine. High casein diet given for first 6 months of life. Tumour cells are seen lining blood vessel. H. & E. × 100.

FIG. 10.—(1306/56). Male rat 28 months after subcutaneous injection of isatidine. Fed on " normal " diet. Hepatoma-like nodule with area of fibrosis at lower left corner of field. H. & E. × 110.

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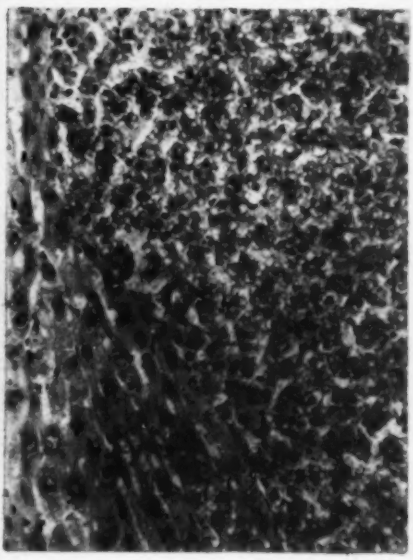

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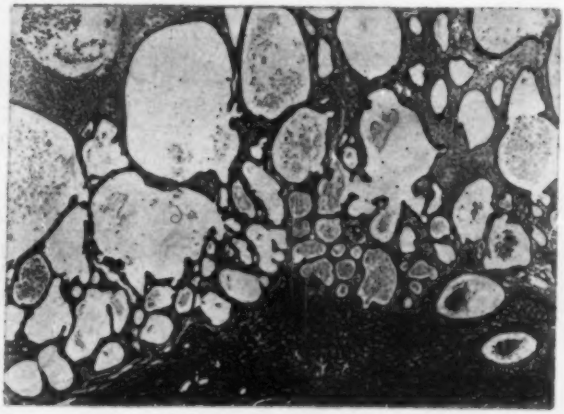

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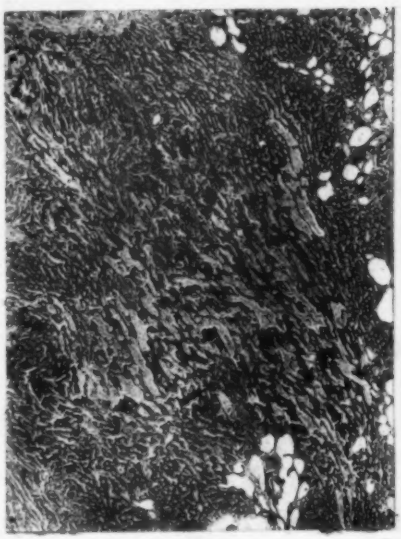
Schoental and Head.



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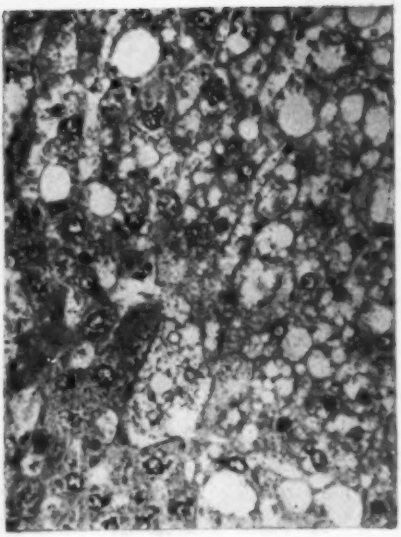

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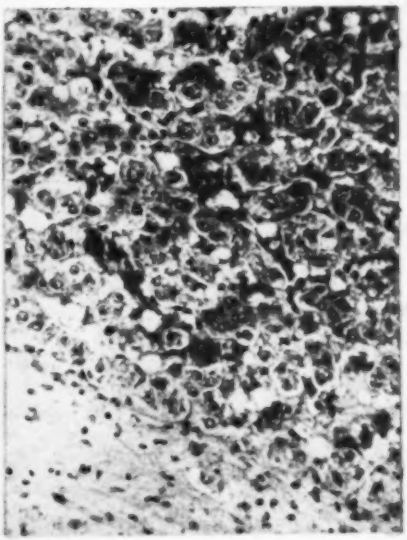

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Schoental and Head.

Two which died after 10 months showed only slight congestion of the liver. Two died after 22 and 23 months and showed thrombosis of blood vessels, dilated sinusoids and cystic spaces containing granular eosinophil material.

The remaining 2 rats showed more severe damage.

The first (812/55) was killed after 11 months and had a large liver with nodules in all the lobes and some cystic areas on the surface. Microscopically the nodules had the appearance of trabecular hepatomata with flattened liver cells around them and no fibrosis. Other nodules were composed of fatty cells (Fig. 8).

The second (744/56) died after 23 months and a large tumour-like nodular mass was present in the left lobe of the liver. Small yellow areas were seen in the other lobes and a cyst in the caudal lobe. Microscopically the lesions appeared to be of endothelial rather than hepatic cell origin and were similar to those described in previous experiments (Schoental *et al.*, 1954) in rats treated with isatidine (Fig. 9). Thrombosed blood vessels were seen and large endothelial cells lining blood vessels. Large cystic blood spaces were also noted.

Of the 6 male rats on normal diet, 3 survived from 12 to 28 months after the last injection. Only congestion of the liver was seen in the 3 which died before 1 year. Two which died after 12 and 19 months showed little change in the liver apart from some congestion of the sinusoids. The third (1306/56) died after 28 months and 2 tumour-like nodules about 1 cm. diameter were seen in the right and median lobes of the liver. Microscopically these were composed of trabeculae of hepatic cells. Some fibrosis was seen around the nodules (Fig. 10).

Of the 6 female rats on normal diet 3 survived from 10 to 18 months after the last injection. The first 2 showed congestion of internal organs, and the third, small nodules, some of which appeared to be early heapatomata. One of these extended through the wall of a portal vein and the cells lined the lumen of the vessel at this area.

(*b*) Three female rats (125–155 g. body weight) received 5 intraperitoneal injections of isatidine, 25 to 30 mg./kg. spread over 2 months and were left without further treatment till they died 18 to 29 months later.

One which died after 20 months showed some small nodules of hyperplasia and a multilocular bile duct cyst. The 2 others showed some cellular infiltration of the portal triads.

Five male rats on the same series died 9–25 months after the last injection. Rat 275/55 died 9 months after treatment ceased and the liver had nodules in all lobes, bronchiectasis and abscesses in kidney and heart wall were present. Microscopically degeneration and fibrosis with hyperplasia of the remaining liver cells were noted. Some liver cells were lying singly and others formed small nodules. Rat 746/55 died 13 months after treatment stopped and had a small cirrhotic liver, 6·5 g. The kidney showed pyonephrosis and the bladder contained 10 stones, the largest 3 mm. diameter, composed of calcium carbonate. Microscopically the liver showed nodular hyperplasia and fibrosis.

Rat 1292/55, killed after 18 months, had a large pale liver with one large nodule in the middle lobe. On miroscopical examination there were nodules of hyperplasia with bands of fibrous tissue around them. One nodule was 5 mm. in diameter and was composed of irregular trabeculae of large hepatic cells and surrounded by flattened liver cells and fibrous tissue.

Rat 340/56 which died 21 months after the last injection had a dark congested liver with a yellow nodule in the left lobe and some haemorrhage into the abdominal

cavity. Microscopically all the organs showed much congestion of blood vessels. An area of infarction was present in the liver and a round nodule composed of congested dilated sinusoids with narrow cords of liver cells between them.

Rat 837/56 was killed 25 months after the last injection and the liver showed cystic and yellow areas. Microscopically a multilocular bile duct cyst and a hyperplastic nodule of liver cells were seen.

Controls

Eight male and 7 female rats were kept as controls and received " normal " diet. Liver biopsies were performed on 2 females aged 7 weeks and 3½ months respectively and no architectural abnormalities were found. All control rats survived from 18 to 33 months and showed no cirrhosis, hyperplasia, or tumours of the liver. Seven had extensive bronchiectasis. Three rats had abscesses in uterine horn, jaw, and middle ear respectively. One had a mammary fibroadenoma, and 1 an ovarian tumour with metastases. Three male rats were given " normal" diet supplemented with a 1 per cent solution of betaine instead of drinking water for 1 year. These survived 22–29 months. No abnormalities were seen in their livers.

DISCUSSION

Rosenberg and Beath (1945) described acute hepatotoxic effects of riddelliine in mice injected intraperitoneally (100–500 mg./kg. body weight), as well as more chronic lesions due to feeding the dried, ground plant of *Senecio riddellii*. In the present experiments riddelliine was tested in rats in which it produced nodular hyperplasia, fibrosis and primary liver tumours. In rats, lesions produced by riddelliine were very similar to those due to other pyrrolizidine alkaloids and developed in animals which appeared in good health during the period of treatment and for many months after the treatment was discontinued. A notable feature was the great variation of the amount of fibrous tissue in livers of individual animals surviving the same time after the treatment with riddelliine and the greater susceptibility of males than females to the action of this alkaloid. The same was observed with the other pyrrolizidine alkaloids, retrorsine and isatidine.

It is well known how difficult it is to diagnose liver tumours and to assess their degree of malignancy, in the absence of metastases (*e.g.* Opie, 1944 ; Magee and Barnes, 1956). Usually authors decide on their own critieria of classification which makes it difficult to compare data from different laboratories. Some more objective critieria are greatly desirable.

The present experiments may serve such a purpose. The treatment was stopped at a time when liver lesions were likely to have developed but were still very slight when examined in biopsy specimens and the animals were allowed to live as long as possible. This procedure showed whether and to what extent the lesions are reversible.

The results show that liver lesions produced by pyrrolizidine alkaloids, retrorsine, riddelliine, and isatidine, are irreversible and progressive. Even in the absence of further treatment the changes present in the livers became more accentuated with time, fibrous tissue if present became more prominent, hyperplastic nodules tended to increase in size, mitotic figures became more numerous

while characteristic large parenchymal cells persisted throughout the lifespan of the animals. Although no metastases were seen in the present series of animals some of the liver lesions were distinctly hepatomata. These were found sometimes in livers in which fibrotic changes were not prominent and also in those in which fibrosis was present. Similar lesions have been shown previously to lead to malignant tumours with metastasis (Schoental *et al.*, 1954) and in view of their progressive nature it seems justified to consider these as neoplastic. However the long survival of the animals in apparent good health does not suggest a high degree of malignancy.

The role of nutrition in relation to liver damaging agents in general (Drill, 1952) and to liver carcinogens such as azo-dyes in particular has been extensively studied (Miller and Miller, 1955). Only a few dietary factors have as yet been investigated in conjunction with pyrrolizidine alkaloids. While protein deficiency has been shown to have a deleterious effect on the survival time of the animals treated with pyrrolizidine alkaloids (Selzer, Parker and Sapeika, 1951 ; Schoental and Magee, 1957) in the present pilot experiment high casein diet favoured longer survival of rats and also the development of liver tumours. While choline deficiency is known to produce liver tumours (Copeland and Salmon, 1946), supplementation of normal diet with choline failed to inhibit the development of tumours due to pyrrolizidine alkaloids (Schoental *et al.*, 1954). It seemed of interest to test whether this lack of action of choline was not due to inactivation of the enzyme, choline oxidase, concerned with the oxidation of choline to betaine, an essential step in the mechanism of transmethylation (Dubnoff, 1949). Asano (1955) reported decrease of choline oxidase in liver tumours.

In a pilot experiment the effect of supplementing the " normal " diet with betaine was therefore tested. The results show clearly that betaine, like choline, failed to inhibit, and may have even favoured the development of liver tumours in our animals. It is of interest to note that rats receiving supplements of betaine were not protected against the more acute changes such as anaemia, subcutaneous oedema, gastro-intestinal haemorrhage, pancreatic abnormalities and the corresponding early liver lesions.

The irreversible character of the liver lesions demonstrated by the present experiments illustrates the dangerous potentialities of pyrrolizidine alkaloids when ingested even in doses which may have no immediate effects on the health of the individual. They also underline the obvious difficulty in tracing the causative agents, when confronted with fully developed chronic liver lesions in animals or men.

SUMMARY

Various liver lesions including liver tumours developed in rats which survived in apparent good health many months after the cessation of a temporary treatment with the pyrrolizidine alkaloids ; riddelliine, retrorsine and isatidine.

Neither high casein diet nor supplements of betaine prevented the development of the various liver lesions.

We wish to thank Dr. P. R. Peacock, the Director of Research, for his interest in this work, Mr. S. Breslin and Mr. C. Bern for the photographs, and the Staff of the Department for their valuable assistance.

For the generous gifts of the alkaloids we are greatly indebted to Professor Roger Adams, University of Illinois, Urbana, (riddelliine), and Professor F. L. Warren, University of Natal, Pietermaritzburg, (retrorsine and isatidine).

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ESTIMATIONS OF CHOLESTEROL IN RELATION TO TUMOURS IN MAN

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THE discovery by Hieger (1946, 1947, 1949) that cholesterol of the highest degree of purity is carcinogenic when injected *sub cutem* in oily media in mice calls for an investigation of the conditions under which this property might take effect in the human body. The question, whether the purest cholesterol is itself carcinogenic, or is the source of a carcinogen, is under examination by Hieger, and by Fieser *et al.* (1955). The following report is of a preliminary nature and is intended to draw attention to some types of human material that are available for such analyses.

METHOD

The fresh tissue was homogenised, washed with water to remove as much blood as possible, dried, powdered and extracted with chloroform in a Soxhlet extractor for about twelve hours. The total cholesterol was estimated on an aliquot of the extract by the Liebermann-Burchard colour reaction, using acetic anhydride and sulphuric acid (Method A, Table I, Column A. Myers and Wardell, 1918). The free and ester cholesterol were estimated on another aliquot, by evaporation of the chloroform, solution in alcohol, and precipitation of the free cholesterol with digitonin. The precipitate was washed with ether and acetone, dried, dissolved in glacial acetic acid, and treated with acetic anhydride and sulphuric acid (Method B, Table I, Column C. Sorbell and Mayer, 1945). The supernatant fluid and ether-acetone washings containing the esters were evaporated to dryness and the residues treated in the same way as the aliquot used for estimating total cholesterol (Method A, Table I, Column B).

CHOLESTEROL IN RELATION TO TUMOURS IN MAN. THE EVIDENCE FROM MORBID ANATOMY

A very brief account may be given here of the observations of some morbid anatomists upon the deposition of cholesterol in relation to cancers in man (for a fuller statement see Kennaway, 1957). Rössle (1943) in a study of 8 scar-cancers of the lung says " an abundant deposit of cholesterol is often to be found in the cancer-free portion of the scar ". Lüders and Themel (1954) described 21 cases arising in association with scars among 74 cases of cancer of the lung. " In almost all cases cleft-like or needle shaped spaces are seen, mostly in the neighbourhood of hyaline scar-tissues . . . these spaces are produced by the dissolving out of cholesterol crystals . . . " Raeburn (1956) in a study of 12 cases of cancer of the lung associated with tuberculous scarring says " These scars are rich in cholesterol ". Dawson (1943, 1948, 1953, 1954) in a series of papers

abundantly illustrated says " Malignant growth in the breast, in my experience, is always initially a duct (intraduct) carcinoma ". She describes " the fatty cyst, as the degenerating cells within it form masses of colostrum-like fatty or foamy cells . . . if fatty material remains for a long time within the ducts it may apparently lead to malignant cell formation and growth, by its action on surviving, viable epithelial cells still lining the duct or cyst . . . In some cases . . . the wall of the cyst is lined by masses of cholesterol crystals . . . " Dr. R. J. R. Cureton of this Department has kindly obtained material from breasts of this type and has described the procedure in the following note : " the hyperplastic changes of " chronic mastitis " are often associated with duct obstruction and retention. In some such breasts there is a particularly abundant secretion of grey, greenish or yellow material, varying in consistency from that of a thin cream to a firm pultaceous material, which may be squeezed out of the ducts in a manner reminiscent of tooth-paste. This condition is commonly spoken of as ' chronic mastitis ' although this term is admitted to be unsuitable. This material has been expressed from the dilated ducts of unfixed breast tissue in amounts varying from 2–5 ml. . . . and treated as described under ' Method ' above, while other specimens have been sent to Professor Fieser ".

Dr. Dawson also writes (personal communication) " I have sections of a breast sarcoma where cholesterol clefts are in an area of dense hyaline tissue in the centre of the tumour, while more peripherally the sarcoma is very active (a fatal case)." Leary (1950) describes crystalline cholesterol esters in cortical adenomas and adenocarcinomas of the human kidney.

RESULTS

This method has been applied to the following materials :

(1) Portions of fresh lungs from 20 autopsies at this hospital, unselected except for the exclusion of cases of cancer of the lung, were washed, dried and powdered,

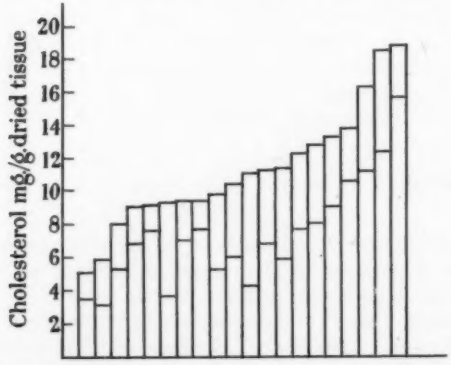

FIG. 1.—The free cholesterol (lower section of column) and ester cholesterol (upper portion) in lungs from 20 autopsies on conditions other than cancer of the lung.

and analysed in triplicate (Table I and Fig. 1). The agreement between the figures for total cholesterol (Column A) and for ester + free cholesterol (Column D) is reasonably good and sufficient for the purpose in view ; hence the investigation

is not intended to be a study of the agreement between the two methods. The means of the two series are almost identical (11·3, 11·4). In 13 of the 20 cases the total cholesterol lies between 8·1 and 12·8 mg./g. dry tissue. The esters make up, on an average, one-third of the total cholesterol, the range being from 20·2 per cent to 59·5 per cent.

TABLE I.—*Cholesterol Content of Portions of Fresh Lung from Cases other than those of Bronchial Cancer*

	mg./g. dry material				
	A. Total cholesterol (estimated by Method A)	B. Ester cholesterol (estimated by Method A)	C. Free cholesterol (estimated by Method B)	D. Ester + free cholesterol (sum of Columns B and C)	Ester as per cent of total cholesterol
	18·6	6·4	12·4	18·8	34·4
	18·9	5·7	15·7	21·4	30·2
	9·1	2·4	6·9	9·3	26·4
	12·3	4·8	7·8	12·6	39·0
	9·5	2·3	7·1	9·4	24·2
	13·9	3·4	10·7	14·1	24·5
	8·1	2·7	5·4	8·1	33·3
	9·5	2·1	7·8	9·9	22·1
	5·1	1·6	3·6	5·2	31·4
	11·1	6·6	4·4	11·0	59·5
	9·3	3·3	7·7	11·0	35·5
	12·8	5·7	8·1	13·8	44·5
	13·3	4·4	9·1	13·5	33·1
	10·5	3·5	6·1	9·6	33·3
	9·4	4·4	3·7	8·1	46·8
	6·5	2·8	3·2	6·0	43·1
	16·3	5·6	11·2	16·8	34·4
	11·3	3·9	6·9	10·8	34·5
	11·4	4·4	6·0	10·4	38·6
	9·9	2·0	5·4	7·4	20·2
Range	5·1–18·9	1·6–6·6	3·2–15·7	5·2–21·4	20·2–59·5
Mean	11·3	3·9	7·5	11·4	34·4
Standard deviation	3·6	4·1	3·2	4·1	—
Standard error of mean	0·8	0·91	0·71	0·92	—

(2) In two cases of bronchial carcinoma, portions of lung from parts not adjacent to the tumour gave figures within the range shown in Table I.

	mg./g. dried material			
	Total cholesterol	Ester	Free cholesterol	Ester + free cholesterol
Case 1	13·6	4·1	10·9	15·0
Case 2	10·7	4·3	5·2	9·5

(3) In some preliminary experiments, estimations of total cholesterol were made upon portions of lung from 10 autopsies upon cases other than those of cancer of the lung after fixation in formalin. These were not washed and gave, as one would expect, higher figures than those in Table I, Column A (mean 19·3, range 8·8 to 26·2 mg. per g.).

(4) Portions of the lungs examined by Dr. C. Raeburn (1956) of Whipps Cross Hospital, Leytonstone, London, E., in his study of scarring of the lung, and of the cancers associated with some of these scars. In all such cases the quantitative result is of course very dependent upon the exact nature of the portion taken for analysis after the specimen for histological examination has been set aside, but this difficulty is unavoidable. Eleven such specimens have been examined of which five were obtained after fixation in formalin had been carried out. Some of these show high figures and further examinations of such material is very desirable, as the microphotographs of Lüders and Themel for instance suggest that the washing out of blood might remove some free cholesterol crystals also. This possibility of loss will be investigated.

	Total cholesterol mg./g.	
	Mean	Range
Tissue from scarred lungs. Washed. Six cases .	16·3 .	5·8–23·5
Formalin-fixed, not washed. Five cases . .	44·5 .	27·7–68·3

(5) Material expressed from mammary ducts of breasts of the type described by E. K. Dawson (see above). We are indebted for these specimens to Dr. R. J. R. Cureton.

TABLE II.—*Cholesterol Content of Material Expressed from Dilated Ducts in Cases of Chronic Mastitis* (*Dr. R. J. R. Cureton*)

	mg./g. dry material				
Case	A. Total cholesterol (estimated by Method A)	B. Ester cholesterol (estimated by Method A)	C. Free cholesterol (estimated by Method B)	D. Ester + free cholesterol (sum of columns B and C)	Ester as per cent of total cholesterol
1 .	62·5	15·0	55·0	70·0	24·0
2 .	76·2	12·3	70·3	82·6	16·1

These preliminary analyses show a very high concentration of cholesterol with a low proportion as ester. Other specimens of this kind have been sent to Prof. L. F. Fieser, of Harvard University, for examination.

SUMMARY

A preliminary account is given of estimations of cholesterol, in the free and ester form, which have been made upon portions of lungs from unselected autopsies upon cases of conditions other than bronchial carcinoma.

Estimations are in progress also upon :

(1) Portions of lungs in the neighbourhood of scarring associated with tuberculosis ; and

(2) material expressed from dilated mammary ducts in cases of chronic mastitis.

I am indebted to Dr. C. Raeburn and Dr. R. J. R. Cureton for a large part of the material used in this investigation which has been supported by grants from the British Empire Cancer Campaign and the Anna Fuller Fund for which I wish to express my thanks.

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EFFECT OF INFLAMMATION UPON THE GROWTH OF TRANSPLANTABLE NEOPLASMS AS DEMONSTRATED BY THE "DOUBLE GRANULOMA-POUCH" TECHNIQUE

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THE effect of inflammation upon the growth of neoplasms has long been the subject of controversy. Some investigators claim that transplantable tumours grow better in inflamed than in normal tissue (Rous, Murphy and Tytler, 1912; Jones and Rous, 1914; Jones, 1926; Carrel, 1925), while others maintain that inflammation does not influence neoplastic growth (Molomut, Spain, Kreisler and Warshaw, 1955), or actually inhibits it (Kubo, 1924; Busch, 1952; Williams, 1944).

According to Kubo (1930), chronic inflammation enhances, while acute inflammatory reactions inhibit the development of neoplasms. Yet, the acute inflammatory reaction that ensues following mechanical trauma to tissue allegedly enhances the growth of subsequent topical tumour implants (Zahl and Nowak, 1949), while chronically repeated mechanical irritation may cause tumours to involute (Aschoff quoted by Hirschfeld, 1919).

The "granuloma-pouch technique" (Selye, 1953)—in which a suspension of transplantable tumour tissue is injected into a subcutaneous air-pouch—appears to be particularly well suited for the study of this problem. It permits the inoculation of transplants into a regularly formed ellipsoid connective-tissue-sac which may or may not have been previously transformed into an inflammatory granuloma by pretreatment with an irritant (e.g., croton oil).

Recently, Hewitt (1956) used the granuloma-pouch technique for his investigations on this subject. He came to the conclusion that, in the mouse, the growth of Sarcoma 37 remains uninfluenced by the acute inflammatory hyperaemia that is induced by formic-acid-pretreatment of the site of inoculation.

In considering this problem, it must be kept in mind that an inflammation caused by irritants which are rapidly destroyed or removed from the site of application (e.g., formic acid, formaldehyde, certain allergens) may largely subside before the tumour implant reaches a critical stage of development. Furthermore, an extensive inflammatory focus may cause considerable stress, with the release of glucocorticoids which delay the growth of transplantable tumours (Selye, 1955). Systemic reactions other than the stress-syndrome and the direct toxic actions of the inflammatory irritant can also influence neoplastic growth. In order to eliminate all these possible complicating factors, it would be desirable to create, in the same animal, two identical transplantation sites, one of which has, while the other has not been transformed into an inflammatory granuloma at the time of tumour inoculation. The "double granuloma-pouch technique" (Selye, 1957) appears to lend itself to this type of study. Here, two tumour-

bearing air-sacs are produced in the same rat and one of these can be transformed into a chronic inflammatory granuloma by the introduction of croton oil into its cavity. Under these circumstances, the toxicity of the absorbed irritant and all systemic reactions to the persistent inflammatory focus become immaterial, because the local effects of the inflammation upon the tumour are compared with an " internal control " in the same individual.

It is the object of this communication to report upon such experiments which indicate that the growth of a transplantable neoplasm is greatly enhanced by a pre-existing topical inflammatory reaction.

MATERIALS

Twenty female Sprague-Dawley rats, with a mean initial body-weight of 100 g. (range : 95–110 g.), were used for these experiments.

In all animals, two air-sacs were prepared as follows : (1) The rat was anaesthetised with ether, the fur of the entire back was removed with clippers and the skin rubbed with nitromersol (" metaphen ") or some other antiseptic. (2) Five ml. of air were injected through a gauge 27 subcutaneous injection needle to form a unilocular sac in the shoulder region. This can usually be accomplished without difficulty, but if connective-tissue partitions tend to subdivide the sac, these can readily be torn with the tip of the needle so as to ensure the formation of a single regularly shaped, round cavity. (3) An identical second air-sac was produced in the lower lumbar region. Immediately following the preparation of these air-sacs, in Group I, a Walker-tumour-suspension plus croton oil were introduced into the cranial pouches, and a Walker-tumour-suspension plus " Mazola Oil " (a brand of corn-oil) were injected into the caudal pouches. In order to control possible variations in the responsiveness of the connective tissue in the shoulder and lumbar regions respectively, in Group II, the croton oil was introduced into the caudal, and the " Mazola Oil ", into the cranial pouch, always simultaneously with the Walker-tumour-suspension.

The tumour-suspension was obtained by mixing one part of an eight-day-old Walker-tumour-256-brei (prepared in a glass homogeniser) with four parts of physiological NaCl solution. All the rats in the two groups received injection of 0·5 ml. of this suspension, in both the cranial and caudal pouches.

As an irritant, 1 ml. of a 1 per cent croton oil solution in " Mazola Oil " was injected into the cranial pouches of Group I and the caudal pouches of Group II, simultaneously with the implantation of the tumour suspension. For control purposes, 1 ml. of pure " Mazola Oil " was introduced at the same time into the caudal pouches of Group I and the cranial pouches of Group II.

Since the proliferating tumour cells themselves produce marked inflammatory reactions in the granuloma-pouch, the rats of both groups were treated subcutaneously with 3 mg. of cortisol acetate microcrystals in 0·2 ml. of water, daily throughout the experiment. Preliminary observations had shown that this amount suffices to depress the inflammatory potential to such an extent that the developing Walker-tumour in itself causes only a very slight inflammatory reaction ; yet, additional local irritation by a 1 per cent croton oil solution overcomes the anti-inflammatory effect of the given dose of cortisol. Thereby, it was possible to exaggerate the difference in the degree of inflammation between the cranial and caudal pouches.

The experiment was terminated after ten days by killing the rats of both groups with chloroform. After the air and inflammatory fluid were removed by aspiration through a syringe, the greatest cranio-caudal and frontal diameters of the collapsed pouches were measured (in centimetres). The product of these two measurements was taken as an approximate, quantitative indicator of tumour development.

RESULTS

Towards the end of the first week, numerous neoplastic nodules became palpable in the cranial pouches of Group I and in the caudal pouches of Group II—that is, at the sites where croton oil had been injected. At this same time, no neoplastic-tissue masses could be palpated in the control pouches in which the neoplasms were in contact with "Mazola Oil" only. On the tenth day, at autopsy, the difference between the inflamed and non-inflamed pouches was striking. In contact with the croton oil, the tumour-cells had proliferated into a thick shell of neoplastic tissue that lined the pouches, while, in contact with "Mazola Oil," only occasional small foci of neoplastic cells were detectable in the linings of the air-sacs (Fig. 1).

Histological examination of the pouch-walls confirmed the macroscopical observations and showed, furthermore, that the proliferation of inflammatory granuloma-cells was also considerably more pronounced in the croton-oil-treated than in the control-pouches of both groups.

The product of the two diameters of the pouches was, in Group I: $15{\cdot}9 \pm 1{\cdot}1$ cm. for the cranial and $5{\cdot}1 \pm 0{\cdot}28$ cm. for the caudal pouches; in Group II: $5{\cdot}8 \pm 0{\cdot}36$ cm. for the cranial, and $10{\cdot}8 \pm 0{\cdot}61$ cm. for the caudal pouches. These figures show that, in both experimental groups, the development of the croton-oil-treated pouches was far superior to that of the others but actually, macroscopical and microscopical examination of the pouch-walls gave an even more convincing proof of this than did the measurements, because the size of the pouch depends partly also upon the development of granuloma-tissue.

We have subsequently repeated these experiments, under essentially identical conditions, on two groups of ten rats each that did not receive cortisol. In the absence of antiphlogistic hormone treatment, tumour development was considerably more rapid in both pouches, but the growth of the neoplasm was still obviously enhanced by contact with croton oil.

DISCUSSION

Although these observations strongly suggest that the inflammatory response to croton oil accelerates the proliferation of transplanted Walker-tumour-tissue, they do not eliminate the possibility that the croton oil might have acted quite

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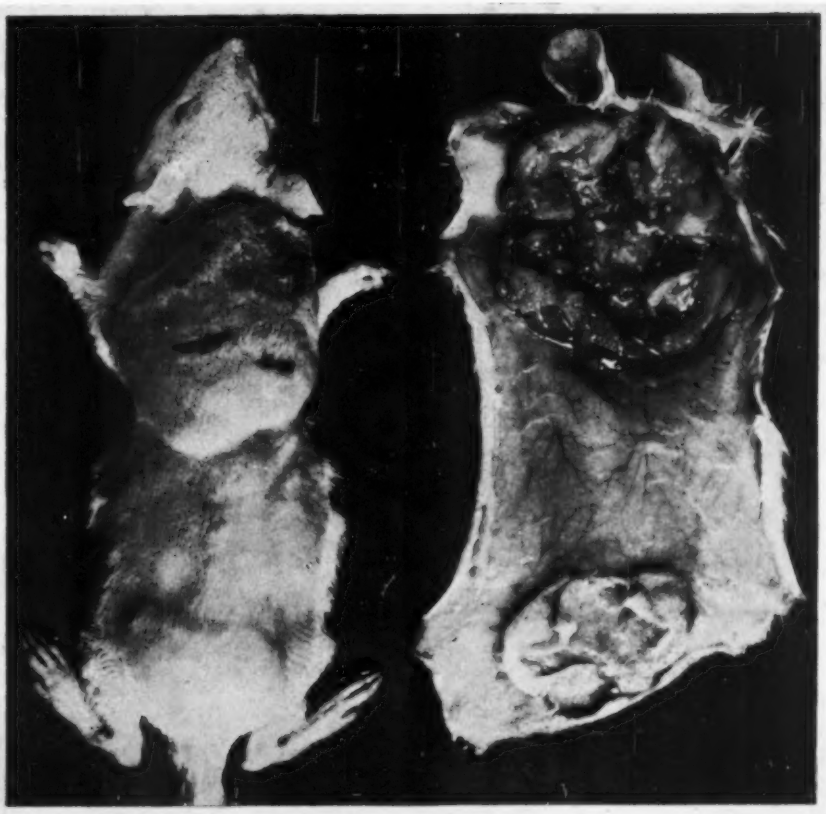
EXPLANATION OF PLATE

FIG. 1.—Effect of inflammation upon the growth of a transplantable tumour as demonstrated by the "double granuloma-pouch" technique. Walker-tumour-suspension was inoculated into both the cranial and the caudal pouch, but, in addition, a strong inflammatory reaction was produced in the cranial pouch by the introduction of croton oil into its cavity. The proliferation of the tumour tissue is much more pronounced in the inflamed cranial than in the caudal pouch. This is clearly seen in the living rat (left), but becomes even more evident after dissection of the skin and opening of the two pouches from the ventral side (right).



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independently of its ability to stimulate inflammation. However, in earlier experiments (Selye, 1957), we showed that if the same amount of croton oil plus Walker-tumour are injected into both air-sacs of a double-granuloma-pouch-bearing rat and cortisol is then introduced into one sac, a marked anti-tumoural effect is manifest only in the pouch into which the hormone is introduced. These experiments were performed to show that glucocorticoids, such as cortisol, inhibit the growth of neoplastic tissue directly by virtue of local effects and not through the intermediary of systemic metabolic actions. However, these same observations also furnish additional evidence in favour of the view that inflammation itself, and not merely contact with croton oil, was the decisive factor in the stimulation of neoplastic growth that we observed in the present experimental series.

SUMMARY

If two Walker-tumour-bearing granuloma-pouches are produced in the same rat and croton oil is injected into one sac only, the growth of the neoplastic tissue is greatly stimulated by the resulting topical inflammatory response. The difference in the development of the tumour in the two pouches can be further accentuated by the subcutaneous injection of cortisol acetate. This is presumably due to the fact that at a suitable dose-level, this hormone almost completely suppresses the inflammatory response to the tumour, but not to croton oil plus tumour.

It is concluded that a topical inflammatory response can greatly enhance the development of transplanted neoplastic tissue.

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The author also wishes to thank the Schering Corporation Limited, Montreal, for supplying the cortisol acetate (hydrocortisone acetate) used in these experiments.

Mr. Kai Nielsen prepared the photograph.

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CHEMICAL INVESTIGATION OF THE NEUTRAL FRACTION OF CIGARETTE SMOKE TAR

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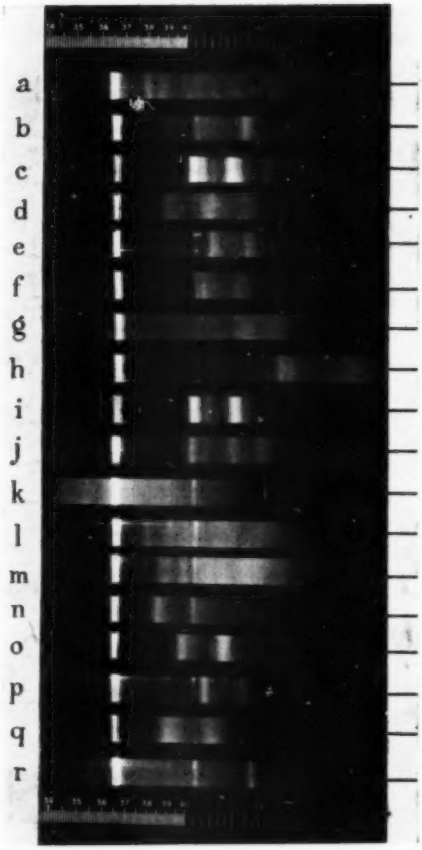
THE first successful production of carcinomata (in the skin of mice) using cigarette tar produced by a method designed to simulate the human habit, was achieved by Wynder, Graham and Croninger (1953). These results were confirmed subsequently by the same workers and by Sugiura (1956) using the same tar which was in all cases obtained from American cigarettes. More recently, Passey, Boyland, Pratt and Hieger (1956), applying a tar prepared from American cigarettes to mice under conditions comparable to those employed by the American workers obtained a much lower incidence of tumours than the latter after the first year of painting. The London workers had two mice presenting papillomata compared with fifteen mice bearing papillomata and two epitheliomata in the American workers' experiment (Wynder, Graham and Croninger, 1953, 1955), the number of animals under test being comparable. A tar, similarly prepared and applied, from British cigarettes, gave negative results after one year. Negative results were reported by Hamer and Woodhouse (1956) in a limited series of tests for carcinogenicity of a British cigarette tar on mouse skin. Similar results were obtained in the authors' laboratory in a series of preliminary experiments designed to test the carcinogenicity for mice of whole and primary fractions of British cigarette tar by (1) feeding experiments (Chalmers, 1954), (2) intra-pulmonary injection (Beck, 1954) and (3) injection into growing embryonic implants (Lyons, Peacock and Peacock, 1956). However, recently Blacklock (1957) produced an oat-celled carcinoma in the lung of one rat of eight given intrapulmonary inoculations of a tar from proprietary filters through which human subjects had smoked.

Therefore it seems, from the latter experiment, that British cigarette tar may be carcinogenic, and (from the comparative series of experiments using British and American cigarette tars) that differences in carcinogenic potency exist between these tars.

Wynder and Wright (1956 and 1957) showed that in the American cigarette tars the carcinogenic factors for mice and rabbits resided mainly, though not exclusively, in the neutral fraction, and further that the 3,4-benzpyrene content was insufficient to account for the biological activity of that fraction.

The present paper records investigations into the composition of the fractions of "neutral tar" from British cigarettes which immediately precede and succeed 3,4-benzpyrene on the chromatographic column, there being evidence from chemical and biological investigations of other tars and oils (see discussion) for the occurrence of carcinogenic agents in these fractions.



	Prominent fluorescence maxima (mμ)	Compound indicated
a	381, 388, 390, 406, 409	1,2-Benzpyrene and unknown compound
b	437	Perylene
c	404, 426, 454	3,4-Benzpyrene
d	388, 412, 435	
e	413, 437	
f	405, 430	
g	434, 464	
h	462	
i	401, 427, 456	11,12-Benzfluoranthene (?)
j	401, 427, 450, 456	„ + 3,4,8,9-Dibenzpyrene
k	343, 352, 363	
l	378, 390, 400	
m	387, 390, 397, 411, 421	
n	385, 407	
o	397, 422, 450	
p	412, 435	
q	388, 412	1,2,3,4,5,6-Tribenzanthracene(?)
r	442, 472	Anthraceno-2′,3′,9,10-Phenanthrene(?)

FIG. 1.—Fluorescence spectra of some compounds isolated from cigarette smoke tar. Solvent: cyclohexane.

EXPERIMENTAL

Five hundred British-type cigarettes were smoked as previously described (Lyons, 1955, 1956), and the smoke products were collected in acetone chilled to − 70° C. Following thrice-repeated extraction of a petroleum ether solution of the whole tar with each of the reagents, dilute H_2SO_4, NaOH and water in order, the neutral tar fraction remaining of weight 4·4 g. was fractionated by adsorption chromatography, using 100–200 mesh alumina (Spence and Co.), and petroleum ether (B.P. 60/80° C.) as eluent initially. The progress of this initial crude fractionation was followed by periodic inspection of the moving fluorescent zones under a filtered ultra-violet lamp. When what was expected to be fluoranthene, as judged from that compound's behaviour on a parallel control column, was about to be eluted, 3 per cent acetone was incorporated into the eluent. This concentration of acetone was increased stepwise to 40 per cent at which concentration the relatively insoluble hexacyclic aromatic hydrocarbons, including 3,4,9,10-dibenzpyrene if present, would be rapidly washed through the column. The chromatography was terminated when little fluorescent material was appearing in the eluates.

Four main fractions were collected, A corresponding with the fluoranthene fraction, B, the expected benzpyrene fraction, and two subsequent fractions, C and D. These were re-chromatographed on alumina and silica gel giving 44 subfractions, which were screened by the fluorescence spectrography method. Some overlapping of zones was seen to have occurred. Following regrouping into twenty fractions, mainly on the basis of fluorescence spectra, each was separately chromatographed on alumina and each resulting subfraction on silica gel, and again spectrographed.

This procedure was repeated a number of times and in all about one hundred fractions screened. Ultra-violet absorption spectrophotometry also was used in attempts to identify the compounds present.

A Hilger E3 Quartz Spectrograph with photoelectric scanner and Cambridge Recorder were used for the fluorescence spectral analysis. Fluorescence maxima were calculated from a mercury spectrum by use of a standard curve derived from the Hartmann dispersion formula. A Uvispek spectrophotometer was employed for ultra-violet absorption spectral analysis.

RESULTS

In the fraction (A) preceding the 3,4-benzpyrene fraction, the following compounds were detected: pyrene, fluoranthene, traces of chrysene and 1,2-benzanthracene, 1,2-benzpyrene and a compound (Fig. 1*a*) with a main fluorescence band at 381 mμ, the absorption spectrum of which was masked by that of the 1,2-benzpyrene. This was followed by a further mixture (Fig. 1*b*) containing both these compounds and perylene, with a fluorescence maximum at 437 mμ.

Chromatography of the benzypyrene-containing fraction (B) yielded an initial eluate which had a fluorescence spectrum suggestive of benzanthracene derivatives. Though many simple derivatives of 1,2-benzanthracene have similar absorption spectra yet it was considered from absorption characteristics that the material here being considered consisted of a mixture of cyclopentane derivatives, 5,6 and 6,7 cyclopentano-1,2-benzanthracenes. Fig. 2 shows the fluorescence tracing of this mixture with 1,2-benzanthracene for comparison.

The fluorescence spectrum of the eluate which immediately preceded that containing the 3,4-benzpyrene (Fig. 1*c*) showed most of the characteristics of that compound apart from a bathochromic shift of 1 mμ and a prominent band at 385 mμ. This is shown in Fig. 3. 1,12-benzperylene was detected by its absorption

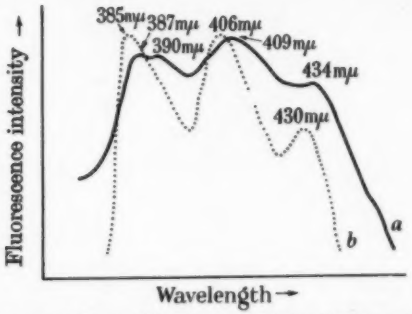


FIG. 2.—(*a*) Fluorescence spectrum of fraction containing 1,2-benzanthracene derivatives. (*b*) Fluorescence spectrum of standard 1,2-benzanthracene. Solvent: cyclohexane.

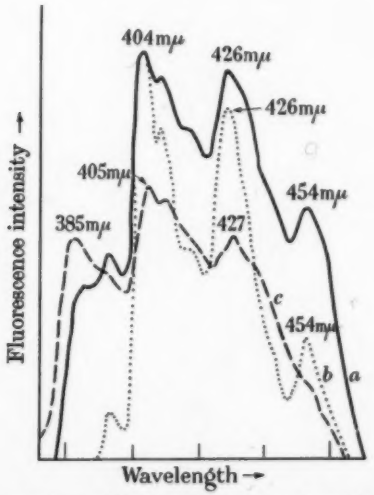


FIG. 3.—(*a*) Fluorescence spectrum of 3,4-benzpyrene from cigarette smoke tar. (*b*) Fluorescence spectrum of standard 3,4-benzpyrene. (*c*) Fluorescence spectrum of fraction immediately preceding, chromatographically, the cigarette tar 3,4-benzpyrene. Solvent: cyclohexane.

spectrum, in the eluate which immediately followed the 3,4-benzpyrene. The fluorescence spectrum is presented (Fig. 4).

The fluorescence spectra shown by subfractions of C and D are presented as they appear on the photographic plate (Fig. 1 *d*–r) in approximate order of elution. Fractions 1*d* and 1*e* both contain the band system 413, 437. Traces of what is

thought to be anthanthrene with a main fluorescence band at 429 was also detected in this fraction. The ultra-violet absorption spectrum in benzene of 1*c* showed peaks at 412, 400, 383, 362, 345, 317, 303 mμ with inflections at 368 and 355 mμ.

Fraction 1*g* gave absorption peaks in benzene at 435, 423, 396, 377, 368, 336, 325, 300 mμ.

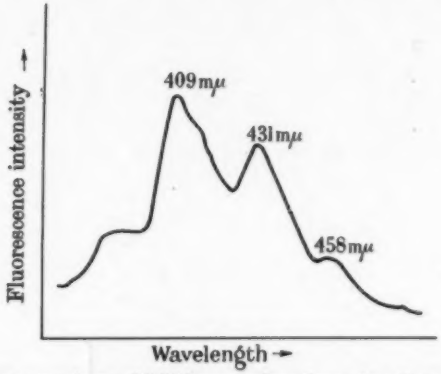


FIG. 4.—Fluorescence spectrum of 1,12-benzperylene from cigarette smoke tar. Solvent: cyclohexane.

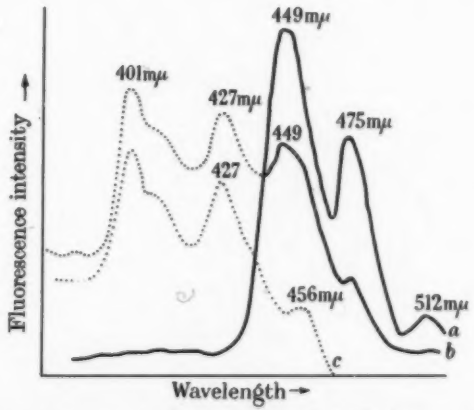


FIG. 5.—(*a*) Fluorescence spectrum of standard 3,4,8,9-dibenzpyrene. (*b*) Fluorescence spectrum of fraction from cigarette smoke tar containing 3,4,8,9-dibenzpyrene. (*c*) Fluorescence spectrum of preceding fraction containing 11,12-benzfluoranthene. Solvent: cyclohexane.

Fraction 1*i* proved identical with a fraction isolated from vehicular exhausts (Compound XIV, Lyons and Johnston, 1957) and detected previously in cigarette smoke (Compound IV, Lyons, 1956). It has been identified as 11,12-benzfluoranthene by its absorption and fluorescence spectra. Fraction 1*j* contains the same compound as well as a new banded system starting at 450 mμ. This latter system is considered to be due to the presence of 3,4,8,9-dibenzpyrene

(Fig. 5). It is present in very low concentration. Its identity was further substantiated by a chromatographic study.

Approximately equivalent concentrations in petroleum ether of 3,4-benzpyrene, 8-Me-3,4-benzpyrene, the benzfluoranthene, 3,4,8,9-dibenzpyrene and 3,4,9,10-dibenzpyrene were mixed and chromatographed on a column of alumina (34 × 1·9 cm.) with increasing concentrations of ether in the eluant. When the 3,4-benzpyrene and the closely following 8-methyl derivative had just been eluted, the 3,4,8,9-dibenzpyrene and the benzfluoranthene compound occupied a zone 7·0–8·8 cm. and the 3,4,9,10-dibenzpyrene a zone 2·5–3·5 cm. from the top of the column. Finally when the benzfluoranthene was eluted, followed by the

FIG. 6.—Absorption spectrum of compound, held to be 1,2,7,8-dibenzfluorene, from cigarette smoke tar. Solvent: cyclohexane.

3,4,8,9-dibenzpyrene, the 3,4,9,10 compound occupied a zone 10·0–12·0 cm. from the top of the column.

The absorption spectrum of this fraction gave peaks which corresponded with those of 3,4,8,9-dibenzpyrene, with further peaks which were suggestive of 1,2,3,4-dibenzpyrene (see Wynder and Wright, 1957).

Fractions 1*k*, 1*l* and 1*m* had absorption characteristics (Bergmann, Fischer, Hirschberg, Lavie, Sprinzak and Szmuszkovicz, 1953) suggestive of the dibenzfluorenes. Fraction 1*l* gave an absorption spectrum indicative of 1,2,7,8-dibenzfluorene (Fig. 6). It is thought that other members of this class of compound are present.

Fractions 1*p* and 1*q*, the latter in particular, give absorption spectra which suggest the presence of 1,2,3,4,5,6-tribenzanthracene. The spectrum of 1*q* is presented (Fig. 7) (*see* Clar, 1952).

Fraction 1*r* shows a fluorescence spectrum and some absorption maxima indicative of anthraceno-2′, 3′,9,10-phenanthrene.

DISCUSSION

The presence of carcinogenic hydrocarbons other than 3,4-benzpyrene has been adduced from biological experiments with certain carcinogenic tars and oils, when it was found that fractions which were demonstrably free of 3,4-benzpyrene were highly carcinogenic and when the concentration of that hydrocarbon was considered to be insufficient to account for the biological activity of the test material. Thus, as an example of the former, Berenblum and Schoental (1947) showed that a coal-tar fraction preceding the 3,4-benzpyrene fraction chromatographically was highly active for rabbit skin, whereas the fraction succeeding

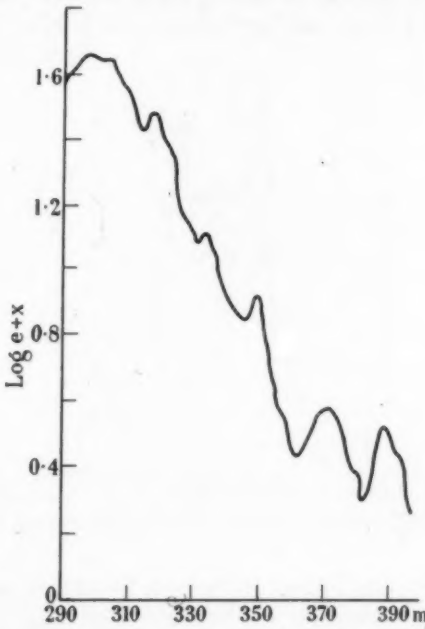


FIG. 7.—Absorption spectrum of compound tentatively identified as 1,2,3,4,5,6-tribenzanthracene from cigarette smoke tar. Solvent: cyclohexane.

benzpyrene was active for both mouse and rabbit skin. As regards the latter point, Poel and Kammer (1957) and Lijinsky, Saffiotti and Shubik (1957) in studying various creosote oils showed that no parity existed between biological activity and benzpyrene content.

Wynder and Wright (1957) showed that a hexane fraction of their neutral cigarette tar which would roughly correspond with the fraction (A) described in the present paper) was more carcinogenic for rabbit skin than for mouse skin, the reverse being true of their succeeding fraction which contained the bulk of the 3,4-benzpyrene and the higher polycycles. In this there is a superficial similarity with the result of Berenblum and Schoental.

In the present work carcinogens were detected preceding 3,4-benzpyrene. Apart from traces of the slightly active 1,2-benzanthracene, 1,2-benzpyrene which is known to be feebly carcinogenic for mice, was detected. Both benzanthracene derivatives have been shown to be unequivocally carcinogenic for mice (Cook, 1931, 1932*b*, 1933*a*; Barry *et al.*, 1935; as quoted by Hartwell 1951). Bonnet and Neukomm (1956) have detected the presence of the same two derivatives in cigarette tar. An unknown compound with a fluorescence maximum at 381 mμ has also been detected in this fraction. We are not aware whether any of the above compounds have been tested on rabbit skin.

1,12-Benzperylene was detected succeeding 3,4-benzpyrene and in similar concentration. This is reported as having slight activity (Cooper and Lindsey, 1955). Berenblum and Schoental have reported a fraction PES-E eluted im-

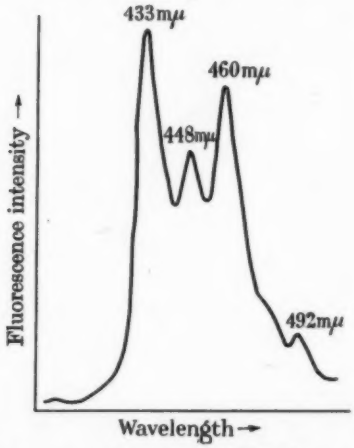


FIG. 8.—Fluorescence spectrum of standard 3,4,9,10-dibenzpyrene in cyclohexane.

mediately after benzpyrene which was potent for rabbit and mouse skin and which had fluorescence bands at 412 and 430 mμ, as well as a more strongly adsorbed fraction PES-G which had a visible fluorescence band at 385 mμ, and which was active for rabbit skin. In the present work fractions were obtained which may be identical with both of these fractions.

The potent carcinogen 3,4,8,9-dibenzpyrene was detected in minute amounts. Bonnet and Neukomm (1956) claimed to have detected minute quantities of 3,4,9,10-dibenzpyrene. This compound has a characteristic fluorescence spectrum (Fig. 8). It was not detected in the present investigation.

1,2,7,8-Dibenzfluorene, detected among other members of this class of compounds, has been shown by Badger, Cook, Hewett, Kennaway, Kennaway, Martin and Robinson (1940) to be carcinogenic for mouse skin.

Some of the compounds detected in the present investigation are shown in Fig. 9.

Approximate concentration levels have been ascertained for the benzanthrance derivatives, the 1,2-benzpyrene, 3,4-benzpyrene, 1,12-benzperylene and 3,4,8,9-

dibenzpyrene. They were found in low concentration, e.g. less than 2 p.p.m. of whole tar, although indications are that concentration levels may be stepped up by increasing the frequency of drawing to a rate unrealistic with respect to the human habit. This latter implication, if true, would disqualify as far as aetiological studies of human cancer is concerned, work in which conditions did not approximate to the normal human method of smoking.

Wynder and Wright (1957) showed their basic fraction to have slight though independent carcinogenic activity. It augmented tumour formation by the neutral fraction. This was especially noted for the less susceptible strain of

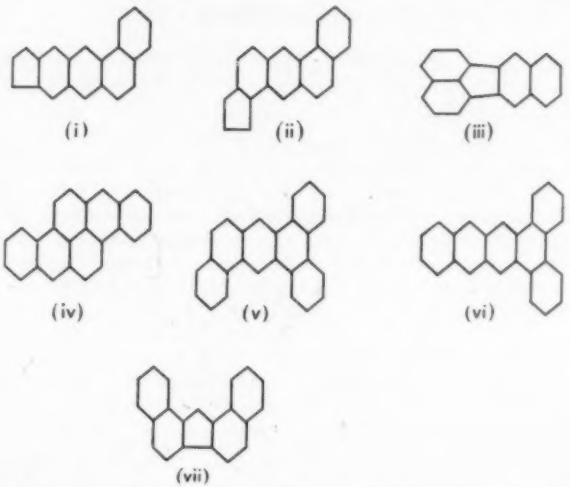


FIG. 9.—Compounds detected in cigarette smoke tar. (i) 6,7-Cyclopentano-1,2-benzanthracene. (ii) 5,6-Cyclopentano-1,2-benzanthracene. (iii) 11,12-Benzfluoranthene. (iv) 3,4,8,9-Dibenzpyrene. (v) 1,2,3,4,5,6-Tribenzanthracene. (vi) Anthraceno-2′,3′,9,10-phenanthrene. (vii) 1,2,7,8-Dibenzfluorene.

mice (CAF_1) in which conditions for assessing promotion phenomena were more likely to have been met.

In the light of the biological and chemical investigations to date the following conclusion seems justified : that cigarette tar prepared in a manner approximating to the human smoking method contains sub-threshold levels of various carcinogens, which, acting in concert, and in the presence of a promoting basic fraction, constitute a feebly carcinogenic tar for rodents.

SUMMARY

An investigation into the composition of a neutral fraction of cigarette tar obtained from British cigarettes in a manner simulating human smoking, was undertaken.

Repetitive adsorption chromatography revealed the presence of compounds, hitherto unrecorded in the present connection, some of which are known to be

carcinogenic for rodents. Attention was drawn to certain fractions containing unidentified compounds which may be carcinogenic.

The carcinogenic agents were present in low concentration, e.g. less than 2 p.p.m. whole tar for those estimated, and this fact is discussed in relation to the biological findings to date.

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SOME BIOLOGICAL FACTORS INCREASING THE RADIOSENSITIVITY OF AN ISOGENIC MAMMARY TUMOUR IN C3H MICE

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IN a previous paper (Cohen and Cohen, 1956), it was shown that a significant radiosensitization of established C3H mammary tumour isografts could be induced by means of a second inoculation of the hosts with radiation-attenuated tumour fragments prior to treatment. It was noted at the time that the best results were obtained with the largest attenuation dose used (5000 r) provided the attenuated inoculum was given *after* implantation of the unmodified tumour graft, and the treatment of the established growth followed at least two weeks after inoculation of the attenuated material. Some questions concerning the optimal attenuation procedure, the possibility of increasing the effect by " booster " inocula, and the specificity of the sensitizing tissue, were not fully investigated. The following series of experiments were consequently designed to elucidate these points.

MATERIALS AND METHODS

Experimental design

Young adult male mice of the C3H/Cg strain were used as tumour recipients in all experiments. In order to maintain as uniform a tumour source as possible, a single autogenous mammary adenocarcinoma, arising in a female of the strain, was serially transplanted for ten generations in a group of mice who constituted a pool of tumour donors for all the experimental groups.

Each of 149 recipient experimental mice was inoculated subcutaneously in the right axillary area with a fragment of the tumour introduced with a trocar and cannula. When the isografts became palpable (about 2 or 3 weeks later), the second, or attentuated, inoculum was given subcutaneously into the left flank. The animals were divided, according to the type of second inoculum received, into the 12 experimental categories listed in Table I.

In two groups, attenuated tumour inocula were prepared from isografts minced with a fine scissors and irradiated with 7000 r and 10,000 r respectively, in a plastic welled slide immediately before implantation. At these dose levels there were no " takes " of any of the irradiated tumour implants.

A further two groups were given repeated " booster " inocula of additional tumour fragments attenuated at the two dose levels. In addition to the first attenuated implant given about three weeks before treatment of the tumour *in situ*, the mice were again inoculated one week before, three weeks after treatment, and at monthly intervals thereafter.

In another category, tumour fragments were irradiated with 7000 r and emulsified in an equal volume of Freund's adjuvant complete with *M. butyricum* suspension (" Bacto-Adjuvant ", Difco) prior to inoculation.

Two groups of mice received cytolyzed tumour material. A tumour was finely minced and suspended in twice its volume of distilled water in an aluminium cylinder for 10 minutes at room temperature. It was then frozen at — 70° C. in a solid CO_2-alcohol mixture for 5 minutes, and thawed in a water bath at 40° C. for 10 minutes. The freeze-thawing procedure was repeated 5 times. A portion of the cytolyzed material was then irradiated with 7000 r, and fragments inoculated into the left flank of one group of tumour-bearing mice, while an unirradiated portion was similarly inoculated into a second group.

Three other groups of tumour-bearing mice received inocula of skin removed from a newborn C3H mouse, finely minced and unirradiated, or irradiated with 7000 r or 10,000 r respectively, prior to implantation.

Another group was inoculated with spleen removed from an adult C3H mouse, minced into fragments and irradiated with 7000 r prior to implantation.

As controls, a number of tumour-bearing mice from the same series, who had received no additional inoculations of any type, were treated concomitantly with the last of the experimental groups.

Finally, some spontaneous tumours were similarly tested using attenuated autografts. The many difficulties in treating autogenous tumours in this strain were discussed in an earlier paper (Cohen and Cohen, 1953*a*), but it was considered important to investigate whether radiosensitization could be induced also in mice bearing tumours that arose spontaneously from their own tissues. Ten female tumour-bearing mice were accordingly selected; small biopsies of the tumour were removed by surgical excision, and irradiated with 7000 r as described above. The attenuated autografts were then re-inoculated subcutaneously by trocar and cannula. The subsequent procedure was exactly as described above for isografts.

The diameters of the tumours at the time of treatment in the 44 mice receiving isografts attenuated with 10,000 r averaged 13·0 mm., with a standard deviation of 4·7 mm. The 72 tumours in the 7000 r attenuation category had a mean diameter of 12·6 mm., standard deviation 3·5 mm. In the 33 controls, the values were 11·8 and 3·0 mm. respectively. There were no perceptible differences in tumour growth rates among the various groups, and each tumour was irradiated *in situ* with 4200 r when it had attained a diameter of about 1 cm., that is four to seven weeks after implantation, and between two and five weeks after the second inoculation. The expectation of cure at this dose, without preparatory sensitization, is about 0·1 per cent, but significant cure-rates can be obtained when host resistance to the tumour has been stimulated by some experimental manipulation (Cohen and Cohen, 1954*b*). Two or more cures in any single experimental group may then be taken as indicative of a significant induced radiosensitization.

Irradiation technique

As in all previous experiments of this series, rontgen rays generated at 240 kVp, no added filters, HVL 0·34 mm. Cu, were used. For irradiation of the tumour *in situ*, the mice were anaesthetized with intraperitoneal pentobarbital, and using a 2 cm. diameter applicator at 25 cm. FSD, the radiation was directed through the tumour retracted on to a wax block at a dose rate of 500 r/min. The attenuation procedure was carried out by irradiation of minced tissue fragments in a plastic welled slide with the same technical factors, but using a 5 cm. diameter

field giving 600 r/min. with full back-scatter, as described in a previous communication (Cohen and Cohen, 1953*a*).

RESULTS

The results of all the experiments involving isogenic tissue inocula, and their radiosensitizing properties, are shown in Table I. The first two items are from the previously published report, and have been incorporated in the table for comparison with the new data.

TABLE I.—*Radiosensitization of the C3H Mammary Tumour Induced by Radiation-attenuated Isogenic Tissue*

Dose *in situ*, 4200 r

Type of inoculum	*In vitro* dose (r)	Number of mice	Number cured at 3 months	Number cured at 6 months	3-month cures (%)
*Viable tumour isografts	3,500	54	7	0	13
*Arrested tumour isografts	5,000	38	19	10	50
Single tumour implant	7,000	11	6	4	
Multiple tumour " boosters "	7,000	10	4	3	
Tumour isograft with adjuvant	7,000	9	5	3	
†Irradiated tumour cytolysate	7,000	10	0	0	39
Irradiated skin isograft	7,000	22	11	6	
Irradiated spleen isograft	7,000	10	2	2	
Single tumour implant	10,000	10	1	0	
Multiple tumour " boosters "	10,000	22	6	2	18
Irradiated skin isograft	10,000	12	1	0	
Total irradiated isografts	—	208	62	27	30
Non-irradiated tumour cytolysate	0	11	0	0	
Non-irradiated skin isograft	0	11	0	0	0
Uninoculated controls	0	11	0	0	
§Total controls	—	33	0	0	0

* From previous data (Cohen and Cohen, 1956).

† This material was cytolyzed, that is dead, before irradiation.

§ To this group may be added 97 unattenuated " controls " from previous data, with no " cures " after 4200 r *in situ*.

Comparison shows that the radiosensitizing effect of attenuated isogenic tissue is critically related to the attenuating dose. Whereas only 13 per cent three-month cures were obtained with an attenuation dose of 3500 r, the cure rate approaches a maximum with doses of 5000 and 7000 r, and then falls off sharply at 10,000 r. Of the three groups, totalling 44 mice, that received inocula attenuated at 10,000 r, only 8 (18 per cent) were cured at three months, while in the corresponding three groups, totalling 43 mice, in the 7000 r attenuation category, 21 (50 per cent) were cured at three months. This difference is significant ($p < 0{\cdot}01$) indicating a loss of radiosensitizing ability with increasing radiation dosage. An analogous loss in immunizing ability of excessively irradiated homografts of Sarcoma-180 has been previously described by Goldfeder (1942).

There is reason to believe that with progressive over-dosage, there is not merely a loss of antigenic power in the cells of the inoculum, but the presence of these heavily irradiated cells may actually stimulate the growth of undamaged tumour

tissue in the host (Revesz, 1956; Scott, 1957) in a manner highly suggestive of the tumour-enhancing properties of lyophilized tissues (Casey, 1934, 1941; Kaliss, 1955). The attenuating efficiency relative to dosage is therefore a biphasic curve whose optimum in the region of 6000 r is probably uniquely characteristic of this host-tumour relationship, and, coincidentally or otherwise, happens to be about the same as the median curative dose for the established tumour irradiated *in situ* (Cohen and Cohen, 1953*a*).

Approximately half of the tumours apparently cured at three months eventually recurred. The injection of " booster " inocula, repeated at intervals over the whole period of observation, did not significantly influence either the initial three-month cure rate, nor did it prevent the late recurrences assessed at 6 months.

Freund's adjuvant was used (at the suggestion of J. B. Graham, personal communication, 1957) in an attempt to intensify the sensitizing effect of the attenuated tumour inocula. Under the conditions of this experiment, however, the procedure did not raise the cure rate materially.

It can also be seen that new-born mouse skin isografts irradiated prior to implantation are just as effective as the tumour isografts at the corresponding doses. This result is, presumably, attributable to the common antigens possessed by the two epithelial tissues (Bashford and Murray, 1906; Maculla, 1948). A non-epithelial irradiated inoculum such as adult spleen seemed to be somewhat less effective in this regard.

One very obvious trend was the complete loss of radiosensitizing ability when killed (cytolyzed) tumour tissue was inoculated, either unirradiated (0/11 cures) or previously irradiated with 7000 r (0/10 cures).

Non-irradiated skin isografts were ineffective (0/11 cures). There were also no cures in the group of 11 uninoculated controls treated towards the end of this series of experiments, indicating that no inherent increase in radiosensitivity had appeared fortuitously during the serial transmission of this tumour line. The over-all three-month cure rate of 30 per cent among the 208 mice receiving irradiated inocula, compared with no cures among the concomitantly treated controls, furnishes highly significant additional evidence of the radiosensitizing properties ascribed to radiation-attenuated isografts.

In the group of 10 spontaneous mammary tumours in female mice that were inoculated prior to treatment with autografts removed by biopsy and irradiated at 7000 r, there were three cures (not shown in the table). This cure rate is not significantly less than that obtained with isografts, suggesting that, in the C3H mouse at least, conclusions derived from the relatively artificial experimental conditions with grafted tumours, are still valid for the truly autogenous host-tumour relationship.

DISCUSSION

All the foregoing results consistently support and reinforce the previously proposed immunological hypothesis (Cohen and Cohen, 1956), that the radiation-attenuated isograft had been rendered antigenic to the host, evoking a generalized systemic immune reaction associated with increased radiosensitivity of the established tumour treated *in situ*. If the hypothesis is correct, then ionizing radiation plays a doubly useful role in this type of investigation: it is able to render autogenous and isogenic tumours and tissues, normally incapable of eliciting any immune response, perceptibly antigenic to their hosts; and, by

measuring induced changes in radiosensitivity, it permits the detection of subliminal resistant states or immunizing factors not ordinarily exhibited in the autogenous or isologous relationship. This method has enabled us to induce and detect what amounts to a " systemic radiosensitizing factor " operating in an isologous host-tumour relationship, analogous to the cellular " mobile resistance factor " described by Prehn, Weaver and Algire (1954) in the case of non-isogenic homologous tumour grafts.

The following facts, however, suggest that the phenomenon of induced radiosensitization cannot entirely be explained in conventional immunological terms: Effective " antigen " is apparently confined to physically intact cells attenuated at critical dose levels ; repeated " booster " inocula fail to modify the result, and addition of adjuvants (Freund's) does not enhance the effect ; some other irradiated tissues are just as effective as the attenuated tumour ; and isologous attenuated tumour antigen seems to be effective only when given to a mouse already bearing a tumour.

Since neither isologous nor autogenous transplants can be considered antigenic in the classical sense, it seems that the attenuated inoculum must contain some new material, or antigen modified by the irradiation procedure, capable of influencing a pre-existing balance between the growing tumour and the resistance factors of the host.

One is led to describe this type of interaction in terms of biological " information theory " (Quastler, 1953), which is peculiarly suited to the dynamics of the situation. Self-regulation of protein synthesis and tissue growth (Weiss, 1952) apparently entails a " negative feedback " cycle regulated through the specific tissue " markers " described by Burnet (1954, 1957). Such a cycle is readily " modulated " by extraneous influences. In this case, inoculation with irradiated tissue amounts to injection of disturbing " information " into the system. Ionizing radiation, with its capacity for random disorientation of protein and nucleic acid structure, could well distort the mechanism of normal information-transfer between isogenic tissue and its host, equivalent, in cybernetic terminology, to the superposition of " noise " or " equivocation " (Yockey, 1956) upon the specific " signal ".

When tissues are irradiated *in situ*, the degree of equivocation, that is the noise : signal ratio, becomes sufficient for the cells to lose their specific identity and hence to be regarded as virtually foreign elements subject to the homograft reaction (Medawar, 1946).

All factors inhibiting reticulo-endothelial function in the host, suppressing the homograft reaction, will retard or prevent radiation responses. Total body radiation which delays the onset of homograft immunity (Dempster, Lennox and Boag, 1950) also reduces the radio-curability of tumours (Cohen and Cohen, 1953*b*), and the corticoid hormones (unpublished data) have been shown to possess a similar action. Conversely, activation of host resistance by exhibition of " equivocated " (irradiated) iso-antigens will facilitate radiation reactions and tumour regression.

It should be noted too that the method of cytolysis, whereby the sensitizing ability of the irradiated isograft is destroyed by repeated freeze-thawing, also inactivates the nuclear antigen responsible for eliciting transplantation immunity with homografts (Billingham, Brent and Medawar, 1956). As an interesting corollary, the latent period of about two weeks before the effects of local irradi-

ation become visible, is the same as that for transplantation immunity and many other sensitization reactions.

In previous experiments (Cohen and Cohen, 1954*a*, 1955) with the C3H mammary tumour grown in genetically alien F_1 hybrid hosts, and with a mutant tumour line in the homozygous mouse, it was shown that the dose of radiation required to cure a tumour treated *in situ* was determined by immunogenetic differences or antigenic diversification between the tumour and its host. This rule now appears, in view of the foregoing discussion and particularly of the current data on induced radiosensitization, to be a special case of a more general law applicable to all irradiated metazoal tissues and tumours: *Radiosensitivity is a function of antigenic equivocation between the irradiated tissue and its host.*

SUMMARY

C3H mice bearing isografts of the C3H mammary adenocarcinoma were given one or more inocula of isogenic tumour, skin or spleen, using structurally intact as well as cytolyzed preparations, some unirradiated and others irradiated with varying doses of rontgen rays immediately prior to implantation. The originally established tumours were then irradiated *in situ* with 4200 r, a dose at which the expectation of cure is normally about 0·1 per cent. The cure rate at this dose level in mice inoculated with living minced isogenic tumour tissue or neonatal skin attenuated in the 5000 to 7000 r range, approaches 50 per cent, indicating a very significant radiosensitizing effect. This attenuating dose is similar to the median curative dose for the tumour treated *in situ* in unmodified hosts, and is critical in that the radiosensitizing efficacy falls off sharply with attenuation doses above or below the optimal range.

Repeated "booster" inocula, or the addition of Freund's adjuvant to the attenuated tumour isografts showed no additional effect. Non-irradiated isogenic skin, and dead (cytolyzed) tumour, whether irradiated or not, were completely ineffective.

A group of female mice bearing autogenous mammary tumours and pretreated with attenuated autografts also showed a substantial cure rate at the test dose.

It seems that irradiation of isogenic or autogenous inocula produces antigens capable of sensitizing established tumours to subsequent irradiation. Consideration of the whole series of experiments leads to the conclusion that radiation acts through equivocation of specific identity markers, evoking a virtual homograft reaction against the irradiated isologous or autogenous tissues.

All the necessary facilities for the maintenance of the animals used in these experiments were supplied at the South African Institute for Medical Research, through the generous co-operation of Professor J. F. Murray. The work was supported in part through the Farquhar Bequest Cancer Research Fund. We wish to thank Mrs. Shelley Jacobson for her invaluable technical assistance.

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THE CULTIVATION *IN VITRO* OF MOUSE ASCITES TUMOUR CELLS. THE EFFECTS OF EXPLANTS OF SOLID TISSUES UPON THE GROWTH OF THE TUMOUR CELLS

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It was inferred from the results of previous researches upon the growth *in vitro* of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells in "pure" culture that the vitality of the cells was limited by the rate at which they lost essential soluble substances to the culture medium (Powell, 1957*a*, *b*). Cultured cells confined in capillaries were able to build up more adequate local concentrations of the protective factors (Powell, 1957*c*). Thus it appeared that the tumour cells were themselves able to synthesize the essential substances or, alternatively, retained protective factors of extrinsic origin more efficiently in capillary than in open coverslip cultures. The observation (Powell, 1957*c*) that single tumour cells survived and divided for relatively long periods in capillaries when cultured together with abundant populations of macrophages was relevant to the problem of the site of origin of the protective factors. It had also been noticed that in coverslip cultures the presence of minute fragments of embryo tissue promoted the growth of neighbouring dispersed tumour cells.

The effects of explants of various tissues upon the growth of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells were therefore studied.

MATERIALS AND METHODS

Mice and tumours.—The ascites tumours were maintained in RIII strain mice as previously described (Powell, 1957*a*).

Tissue cultures.—The general methods, including preparation of media components, selection of ascites tumour cells and spatial arrangement of cultured cells, were similar to those used previously (Powell, 1957*a*). The double-coverslip method of culture was used exclusively. In each experiment "pure" cultures of ascites tumour cells were employed as controls to cultures containing explants placed within the colonies of tumour cells. The distributions and numbers of tumour cells were comparable in the cultures of the two series. Observations were made on the effects of each kind of explant on both strains of malignant cells.

In the course of the experiments it was found that the degree of protection given to the tumour cells by the explants varied with the treatment of the latter. Explants used in definitive experiments were prepared in a mixture consisting of 40 per cent ascitic tumour or horse serum and 60 per cent of Earle's balanced saline solution. The saline solution usually included a proportion of embryo extract. The explants were 1–2 mm. in diameter and cut to size as soon as possible

before use from larger pieces of tissue which were stored in the saline-serum solution. The cultures were set up as quickly as possible to avoid deterioration of the tumour cells and explants. Only the explants for each set of 6 identical cultures were prepared at one time and they were stored in a minimal quantity of medium. Explants exposed to saline solutions for more than a short time were only feebly protective.

The standard culture medium consisted of equal parts of either chick or mouse embryo extract and ascitic fluid diluted as required with a mixture of equal parts of fowl and either ascitic tumour or rat plasma. The replacement of chick with mouse embryo extract or of ascitic tumour with rat plasma, in the diluting mixture, had no significant effects on the fates of the cultured cells. Medium and low population densities of tumour cells were used in order to evaluate the protection given to them by explants. The selected final densities of tumour cell population in the cultures were obtained by appropriate dilutions of the cellular ascitic fluid. These densities were related to average intercellular distances between tumour cells expressed as cell diameters. Tumour cells at a medium density were spaced 2–5 diameters apart and at a thin density were separated by 5–10 diameters. When they were about 1–2 diameters apart their mutual protection obscured the early effects of the explants.

At intervals of 48 hours cultures were rinsed in Earle's solution and either fresh culture medium or nutrient medium, consisting of 40 per cent horse or ascitic tumour serum and 60 per cent of dilute embryo extract, applied. When nutrient medium was not used a small drop of Earle's solution was placed in the well of the cavity slide. It was necessary to retain the original plasma clot in order to study the effects of the explants upon tumour cells. Although cultures began to degenerate by about the 5th day this was unimportant in view of the short survival of ascites tumour cells in " pure " cultures.

Dispersed ascites tumour cells were easily distinguished from the normal cells in the cultures. Their shape, size, large nuclei with irregular chromatin bodies, and pronounced basophilic reaction of both cytoplasm and nuclei, were characteristic. Their distribution in the combined cultures, and comparison of " pure " cultures, combined cultures, and cultures of explants alone, also aided in the identification.

Cultures were fixed in Heidenhain's " Susa " mixture at daily intervals and stained with diluted Harris' haematoxylin. They were mounted in the reversed position and covered with No. 0 coverslips. Small explants were advantageous for making the permanent preparations.

EXPERIMENTAL RESULTS

The duration of survival and incidence of mitosis in the control " pure " cultures of ascites tumour cells in the present experiments were comparable to those reported earlier (Powell, 1957*a*) and will not be discussed in detail here. The majority of the tumour cells in cultures with medium population densities survived 24 hours though dividing cells were few. In cultures with low populations most of the tumour cells degenerated in this period. After 48 hours the denser cultures contained a majority of degenerating cells. The effects of the explants on the two strains of tumour cells were closely similar and have therefore been described collectively.

Combined cultures of embryonic chick heart and ascites tumour cells

Since the culture medium contained both fowl and mammalian plasma both tumour and chick cells were provided with suitable nutriment. Explants of 11-day-old embryo heart were used.

Within 24 hours the explants had developed halos of wandering cells and fibrocytes had begun to grow out. Tumour cells within 1–5 cell diameters of the explants were observed in division in cultures of both medium and thin population density (Fig. 1). The cells near explants were viable and usually epithelioid, although transformed cells of both tumour strains were sometimes seen. Even in cultures with medium population densities dividing tumour cells were rarely seen away from the explants. Tumour cells some distance from explants tended to degenerate, especially in the thin cultures.

After incubation for 48 hours, while the outgrowths of fibrocytes had increased in size, no tumour cells in division were found but those in contact with (Fig. 2) and adjacent to (Fig. 3) explant cells were healthy. Malignant cells placed away from explants had degenerated (Fig. 4). At 72 hours the cultures had the same general appearance and most surviving tumour cells had been overgrown by fibrocytes. These tumour cells were viable.

On comparing the control and explant cultures it was evident that the dispersed tumour cells had benefited from the presence of the heart explants. The protection was limited to the immediate vicinity of the explants and was more effective in respect to survival than to division of the tumour cells. This limited protection was possibly due to the heterologous relation between chick and mammalian tumour cells as mouse tissues were later found to give much greater protection.

Combined cultures of embryonic mouse muscle and ascites tumour cells

Explants were prepared from thigh muscle of embryos of about 14 days' intrauterine growth. In complete contrast with the "pure" cultures, tumour cells in combined cultures grew and divided freely up to the 5th day of cultivation even when widely separated from each other and the explants. The protective effects of the explants was inversely proportional to the distance between them and

EXPLANATION OF PLATES

Photomicrographs of ascites tumour cells cultured *in vitro* in the presence of explants. Fig. 2–4 were taken from the same preparation.

FIG. 1.—Sarcoma 37 cells cultivated in the presence of an explant of embryo chick heart for 24 hours. Dividing tumour cells of inner zone near explant.

FIG. 2.—Sarcoma 37 cells cultivated in the presence of an explant of embryo chick heart for 48 hours. Tumour cells of inner zone at edge of outgrowth.

FIG. 3.—Sarcoma 37 cells cultivated in the presence of an explant of embryo chick heart for 48 hours. Tumour cells of middle zone.

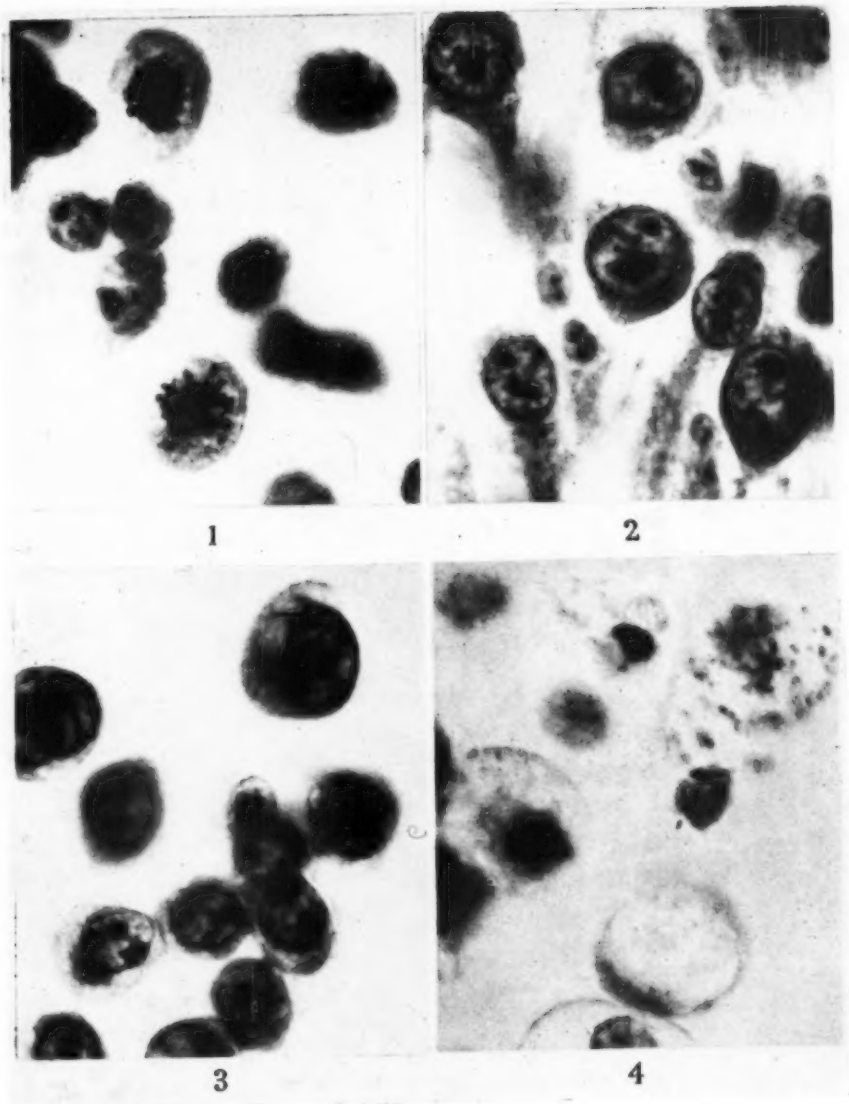
FIG. 4.—Sarcoma 37 cells cultivated in the presence of an explant of embryo chick heart for 48 hours. Tumour cells of outer zone.

FIG. 5.—Sarcoma 37 cells cultivated in the presence of an explant of mouse embryo skeletal muscle for 48 hours. Tumour cells near fibrocyte outgrowth.

FIG. 6.—Ehrlich carcinoma cells cultivated in the presence of an explant of mouse embryonic membrane for 72 hours. Inner zone of dividing cells.

FIG. 7.—Ehrlich carcinoma cells cultivated in the presence of an explant of mouse embryonic membrane for 72 hours. Group of tumour cells of inner zone.

FIG. 8.—Sarcoma 37 cells cultivated in the presence of an explant of mouse lymph node for 72 hours. Group of tumour cells from inner proliferating zone.



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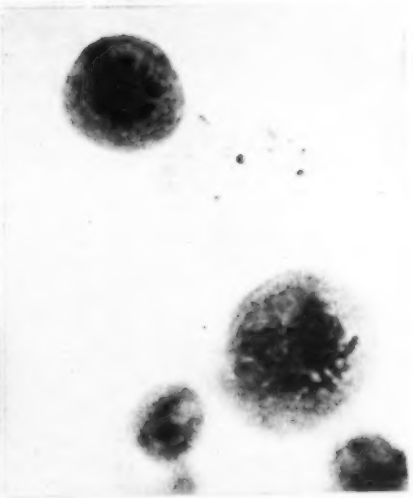

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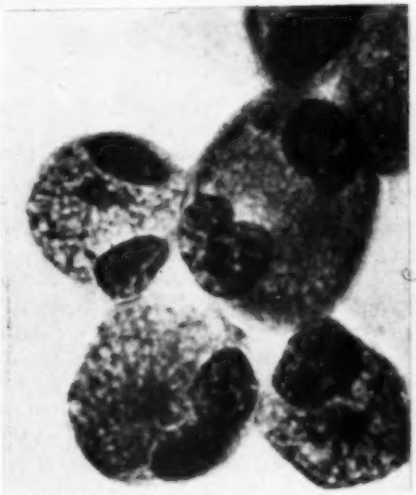

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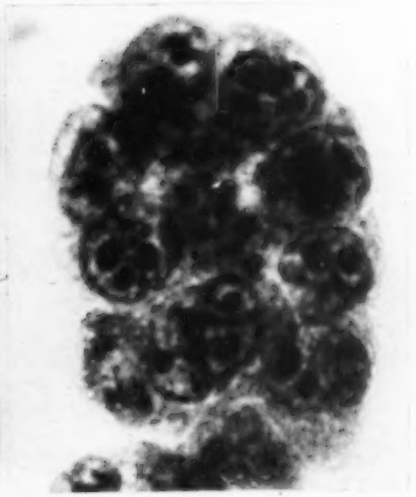

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the dispersed malignant cells and varied with time. Protection of tumour cells placed at the margins of the coagula was evident in cultures incubated for 24 hours but thereafter diminished.

Three zones of graded protection were distinguished: a peripheral zone, in which tumour cells were protected for at least the first 24 hours; an inner zone a few millimeters in width immediately surrounding the explant, in which the tumour cells grew and divided well; and an intermediate zone, in which the cells survived for significantly longer than 24 hours but rarely divided. The protection given by the normal to the malignant cells was independent of physical contact between them.

Cells in the outer zone were usually necrotic by 48 hours. Those in the inner zone flourished until they were obscured by the fibrocytic outgrowth (Fig. 5). Ones in the inner part of the middle zone benefited by the approach of the outgrowth but the outer tumour cells tended to die as the cultures aged. Occasionally very widely separated tumour cells were protected by explants over the critical first day of incubation.

Mouse embryo muscle gave much better protection than embryo chick heart to dispersed tumour cells. The protective effect diminished some 24 hours after setting up the cultures, i.e., before outgrowths of fibrocytes were well established. Thereafter the rate of production of the beneficial factors appeared to be more or less constant and tumour cells adjacent to explants were protected as long as the explants were themselves healthy. Tumour cells overrun by fibrocytes remained vigorous and divided.

Combined cultures of mouse embryonic membranes and ascites tumour cells

Membranes were taken from RIII strain mice at about the 14th day of pregnancy. The allantochorions from almost full term mice were stretched too thinly to be easily separated from the foetuses and manipulated. Those from younger embryos were too delicate for convenient handling. Before preparing explants the membranes were thoroughly washed in Earle's solution.

Explants of the membranes grew luxuriantly. Outgrowing cells appeared in small numbers within 24 hours. Wandering cells were not abundant. The early outgrowths were mesenchymatous. Later, well-developed epithelial sheets and monocytes made their appearance.

Dispersed ascites tumour cells remained viable for the first 24 hours of cultivation but the few mitotic cells were restricted to the environs of the explants. Tumour cells in this region were vigorously healthy and polymorphic. Away from explants the malignant cells were usually rounded in outline. Coherent pairs of tumour cells were common and appeared to be daughter cells which had stayed in contact after division.

After cultivation for 48 hours the general picture had changed with the increased growth of normal outgrowth and malignant cells. The latter showed an increased incidence of mitoses and of cell aggregates. The groups of tumour cells had also increased in size by division. The tumour cells near explants had markedly increased in number over the second day of culture. The concentric zone of actively growing tumour cells was some 3–4 mm. in diameter. The distribution of groups of tumour cells and the changed incidence of groups with particular numbers of cells indicated that the aggregates had been formed by coherence of

successive daughter cells. The tumour cells were usually epithelioid. In 48-hour-old cultures the outermost tumour cells had begun to degenerate.

In cultures maintained for 72 hours the distinctive demarcation of the tumour cell colony into peripheral degenerated and inner growing cells (Fig. 6) had become pronounced. Between these zones was one of viable but non-proliferating cells (Fig. 7). Aggregates of tumour cells now included groups of up to 12–15 cells. By the 4th day groups of about 30 cells were found but by this time the zone of growing and dividing tumour cells was largely overwhelmed by the outgrowth of fibrocytes and epithelial cells from the explants. Malignant cells embedded in the outgrowths were healthy and seen in mitosis. The outgrowth had now reached the outer limit of the original zone of growing tumour cells. Dividing tumour cells were present in reduced numbers in 96-hour-old cultures. At 5 days the cultures began to degenerate but dividing tumour cells were still seen.

Washing the cultures in Earle's solution and renewing the medium stimulated the growth of the normal tissues and, presumably indirectly, of the tumour cells. In young cultures dividing tumour cells were almost invariably smoothly rounded. In older cultures the tumour cells in the later phases of mitosis indicated by their shape and surface irregularities that they were of the more differentiated rather than of the epithelioid form (Powell, 1957*b*). Aggregated cells of both tumour strains were usually epithelioid. Isolated cells were more commonly modulated. Completely isolated tumour cells divided freely.

The explants of mouse embryonic membranes stimulated the growth of the malignant cells more than did those of other tissues tested. Extracts of the membranes also stimulated the growth of explants of various tissues. It is possible that the stimulatory products of the membranes may be available to developing embryos.

Combined cultures of thymus gland and ascites tumour cells

Explants were prepared from the glands of adult RIII strain mice. After incubation for 24 hours the explants were surrounded by vast numbers of small wandering cells. These were mostly small lymphocytes and few large lymphocytes or monocytes were seen. After 48 hours monocytes were common. Forms transitional between monocytes and macrophages were abundant.

In 24-hour-old cultures the tumour cells were found to be mostly healthy and especially vigorous within 1–2 mm. of the explants. Degenerated tumour cells were present and most numerous towards the edge of the coagulum but viable cells occurred throughout its area. Dividing cells were frequent and concentrated within a distance of about 1 mm. of the explants. They always retained a smooth external surface.

Mitotic tumour cells were observed close to explants in 48- and 72-hour-old cultures, but decreased in frequency with increasing age of the cultures. Tumour cells near groups of monocytes but outside the effective range of the explants remained healthy. After the 96th hour of cultivation the rate of degeneration of the tumour cells rapidly increased. They remained healthy for the longest periods in the vicinity of explants. Surviving tumour cells in older combined cultures were much larger than those originally inoculated and became relatively differentiated. Monocytes were especially abundant on the surfaces of coverslips in areas of liquefied plasma.

Definite evidence of migration of tumour cells was not found. Aggregates of epithelioid tumour cells developed and appeared to be due to the coherence of successive generations of cells. Both viability and mitotic incidence were determined by distance from the explants. Viable cells extended further from explants than did mitotic tumour cells. Accumulation of cell débris and waste products finally caused the cultures to degenerate.

Combined cultures of lymph node and ascites tumour cells

Inguinal lymph nodes of adult RIII strain mice were used. After incubation for 24 hours the explants were surrounded by very numerous small amoebocytes which were mostly lymphocytes. Although at this time the incidence of degenerated tumour cells in thinly populated cultures increased somewhat towards the periphery of the colony, the great majority were viable. Dividing cells were common and concentrated in a concentric zone about 1 mm. in width around the explant. The halo of wandering cells encompassed this zone of frequent mitoses.

During the 2nd day of incubation the output of amoebocytes increased and monocytes were occasionally seen. The latter were less common than in corresponding spleen cultures. The numbers of necrotic cells increased between 24 and 48 hours of incubation, particularly away from explants. Dividing tumour cells were common until after the 4th day of cultivation, though found in progressively decreasing numbers.

Small aggregates of malignant cells were formed near explants (Fig. 8). The protective effects of the explants on the tumour cells were well marked and concentrically zoned as with explants of other tissues.

Combined cultures of spleen and ascites tumour cells

The spleens of adult RIII strain mice were used. During the first 24 hours of cultivation large numbers of small wandering cells and occasional monocytes emerged from the explants. The latter were not abundant until after the 2nd day. Numerous mitotic tumour cells were present within 2 mm. of the explants in 24-hour-old cultures. Most tumour cells were healthy but degenerated cells occurred and were common at the margins of the colonies.

Small aggregates of 3–4 tumour cells developed near explants in 48-hour-old cultures although single tumour cells predominated. The coagula often liquefied by this time. In 72-hour-old cultures monocytes began to be the predominant form of amoebocyte. Dividing tumour cells were still frequent but were fewer than before. Malignant cells close to explants and within the region occupied by monocytes were healthy at this time. Externally to this region the tumour cells had usually degenerated. In 96-hour-old cultures the small wandering cells were mostly replaced by monocytes and these enabled neighbouring tumour cells to divide. Most of the malignant cells had tended to degenerate by this time.

Combined cultures of solid tumours and ascites tumour cells

Ehrlich carcinoma and Sarcoma 37 tumours maintained in RIII strain mice were used. They had been derived from the ascites forms by subcutaneous injection of ascitic fluid.

The tumour explants caused liquefaction of the coagula. This made accurate comparison of the relative protective capacity of the tumour and other explants difficult. It inhibited the development of intact outgrowths and caused losses

of cells in subculturing. However, it was clear that dispersed tumour cells were enabled to survive and divide in proximity to the explants.

The tri-zonal arrangement of ascites tumour cells, namely an outer zone of rapidly degenerating cells, a middle zone of cells which remained viable for several days, and an inner zone of dividing and growing cells was clearly evident.

In regions where the plasma has liquefied it was not possible on cytological grounds to distinguish between tumour cells derived from the explants and ones from the ascitic fluid. The explant tumour cells became separated and rounded up in the fluid medium. Such cells were seen in mitosis. The solid tumours were homologous with the ascites tumour cells in the combined cultures but it was also observed that sarcoma 37 explants protected Ehrlich carcinoma.

DISCUSSION

Explants of a variety of tissues were found to protect dispersed ascites tumour cells of both strains against the deleterious effects of comparative isolation. In view of the distances between the widely separated tumour cells and the explants the protection appeared to be due to soluble substances diffusing from the latter through the medium. Three concentric zones, which differed in the degree of benefit afforded to the tumour cells were distinguished. These zones were not sharply demarcated *inter se*. They are illustrated (Fig. 1–4) with reference to explants of embryo chick heart. Tumour cells of the inner zone nearest the explant divided freely in 24-hour-old cultures (Fig. 1). In 48-hour-old cultures cells of this zone were overgrown by chick fibrocytes, they remained healthy but did not divide (Fig. 2), cells of the middle zone were viable (Fig. 3) while those of the outer zone were mostly necrotic (Fig. 4).

In cultures of tumour cells with explants of mouse tissues, which were more protective than those of chick heart, the zonation of the tumour cells was clearer.

Tumour cells in the outermost and widest zone survived for periods significantly longer than in comparable "pure" cultures but which were nevertheless still limited. In this zone mitoses were virtually absent after relict divisions had been completed. In the innermost zone, extending 1–2 mm. from the explants in favourable conditions, tumour cells grew and divided progressively until the explants degenerated or the cells were covered by outgrowth. Healthy and dividing cells were seen enclosed by the latter. In the intermediate zone the malignant cells survived longer than those in the outer zone and dividing cells were commoner but growth was not progressive. Cells in this region were stimulated by the approach of outgrowth.

The protective effects on the tumour cells were inversely proportional to distance from the explants. This relation suggested a concentration gradient of essential substances decreasing in a radial direction from the explants. The area of protection reached its maximum size within the first day of cultivation. Thereafter it decreased in area. The protective factors diffused through the coagulum to reach its margin, a distance of approximately 1 cm., within the first 24 hours. Explants killed or reduced in vitality by overlong exposure to saline solutions had comparatively feeble powers of protection. From comparisons of cultures of similar material it was seen that the degree of protection given by explants was related to their vigour of growth. The temporary protection of peripheral tumour cells indicated that explants liberated the greatest amounts of the essential substances soon after the cultures were set up.

The topographical correlation between the progressive growth of the innermost tumour cells and the outgrowths from explants in cultures 2 or more days old pointed to a continuous output of diffusible substances but at a lower level than that of fresh explants. The "residual growth energy", in Carrel's sense, of explants in non-nutritive media may be related to the amounts available of such diffusible substances.

Survival of tumour cells required smaller amounts of diffusible substances than did growth and division, assuming a quantitative rather than a qualitative difference in these substances at different points in a diffusion gradient. There were no marked qualitative differences between the effects of the protective substances liberated by the various kinds of explants tested. The rôle of amoebocytes present in the explants was perhaps important.

The explants had little effect on the morphology of the malignant cells. These tended to be epithelioid but transformed cells were present and sometimes radially orientated in respect to the explants. Such orientation was commoner in older cultures.

The protective factors were soluble in Earle's solution. They were probably heterogeneous and akin to or identical with those responsible for the growth-promoting effects of embryo extract. It appeared that the maintenance of a certain minimum concentration was essential for life and that greater amounts stimulated growth and division. The loss of these factors by isolated cells was possibly secondary to altered permeability of cell membranes.

While it was evident that the malignant cells benefited from the presence of normal embryonic cells, lymphoid tissues, embryonic membranes, and explants of solid tumours, the initial origin of the protective factors was not clearly apparent. The explants were heterogeneous histologically and the only type of cell definitely shown to form the factors was the monocyte. Amoebocytes were present in all the explants tested. The function of these cells in promoting the growth of malignant cells required further investigation.

SUMMARY

The protective effects of explants of various tissues upon the vitality of dispersed Sarcoma 37 and Ehrlich carcinoma ascites tumour cells in plasma cultures are described.

It is concluded that the protection given by the explants is due to soluble substances which diffuse from them through the medium. The concentration of protective factors is greatest in the immediate vicinity of the explants and decreases radially to the edge of a culture. This concentration gradient is responsible for a concentric tri-zonal arrangement of the dispersed tumour cells of the colony is respect to their vitality.

The significance of the protective factors is briefly discussed.

I am grateful for the assistance of Mr. G. A. Butcher with this work. The expenses of these researches were defrayed from a block grant by the British Empire Cancer Campaign.

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ENZYME ACTIVITY IN RELATION TO CANCER

Inhibition of Urinary β-Glucuronidase of Patients with Cancer of the Bladder by Oral Administration of 1 : 4-saccharolactone and Related Compounds

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A number of clinical and statistical observations led to the suggestion (Boyland, Wallace and Williams, 1955) that patients suffering from cancer of the bladder excrete carcinogenic substances in the urine. The investigation of Bonser, Bradshaw, Clayson and Jull (1956) and of Allen, Boyland, Dukes, Horning and Watson (1957) indicate that a number of *ortho*-aminophenols can induce cancer in experimenta lanimals. Two *ortho*-aminophenols, both metabolites of tryptophan, are normally present in urine and these substances, 3-hydroxyanthranilic acid and 3-hydroxykynurenine, have produced tumours when implanted in the bladders of mice (Allen *et al*, 1957). Both 3-hydroxyanthranilic acid and 3-hydroxykynure-

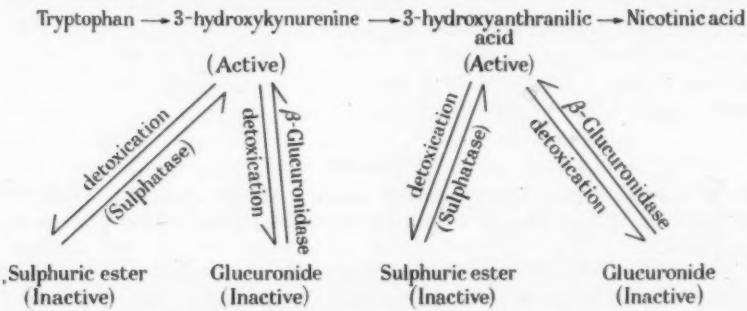


Fig. 1.—The equilibrium between naturally occurring *ortho*-aminophenols and the corresponding detoxication products.

nine were shown to be present in increased amounts in the urine of patients suffering from cancer of the bladder (Boyland and Williams, 1954, 1956). *ortho*-Aminophenols are usually excreted as sulphuric esters or as glucuronides in urine and these compounds would be expected to be inactive as carcinogens unless hydrolysed by enzymes. The carcinogen concentration would be dependent on the time which the urine remained in the bladder, on the concentration of *ortho*-aminophenol conjugates in the urine and on the urinary activity of the enzymes sulphatase and β-glucuronidase (Fig. 1). The activity of both sulphatase and β-glucuronidase in urine is raised in cancer of the bladder (Boyland, Wallace

and Williams, 1955) but urinary sulphatase hydrolyses the sulphuric esters of some carcinogenic aminophenols only slowly (Boyland, Manson, Sims and Williams, 1956) and therefore β-glucuronidase must play a part in the hydrolysis of such conjugates. If the urinary β-glucuronidase activity in patients, in which new tumours would be expected to develop, could be inhibited, then these tumours should be prevented.

The present work deals with the urinary β-glucuronidase activity of patients suffering from cancer of the bladder and the effect of treatment with β-glucuronidase inhibitors on the enzyme activity. The inhibitors used are all derived from glucosaccharic acid, which have been previously shown to inhibit β-glucuronidase *in vitro* (Karunairatnam and Levvy, 1949; Mills and Paul, 1949; and Levvy, 1952). Saccharo-1 : 4-lactone (glucosaccharo-1 : 4-lactone) is the most effective inhibitor and the inhibitory action of saccharates used in these investigations is probably due to the 1 : 4-saccharolactone which they contain or is produced from saccharate in the body. Saccharic acid is readily and reversibly converted to a mixture of saccharo-1 : 4-lactone and saccharo-3 : 6-lactone.

Saccharo-1 : 4-lactone (II) is formed from saccharic acid (III) by loss of the elements of water. This dehydration reaction which is reversible gives the lactone (II) which has a structural resemblance to glucosiduronic acids (*cf.* I). Glucosaccharo-1 : 4-lactone is probably an active inhibitor of β-glucuronidase because of this similarity in structure which allows it to occupy the enzyme centre which is concerned with β-glucuronidase activity. Glucosaccharo-3 : 6-lactone (IV) which is also formed from saccharic acid and only differs from the 1 : 4-lactone, in the position of hydroxyl groups is a poor inhibitor. (For formulae I to IV see page 585).

EXPERIMENTAL

The β-glucuronidase activity is almost always increased in the urine of patients with cancer of the bladder and remains high in most cases even after the tumour has been removed (Boyland, Wallace and Williams, 1955). The method of estimation has since been modified (Boyland *et al.*, 1957) in the following ways—(*a*) A 20 per cent solution of thymol in benzene is used as a preservative as it is more effective than the benzene used in the original work. (*b*) The buffer concentration has been increased so that the activity of alkaline urine specimens (previously discarded) may also be estimated. The enzyme is unstable in strongly alkaline solution. The upper limit of the normal range using this method is 1·2 units/ml. All β-glucuronidase values are expressed as units of activity per ml. of urine. The unit of activity is defined as being that which liberates 1 μg. of phenolphthalein per hour at 37° C. In all cases the total 24 hr. output of urine was collected and the test performed on a 1 ml. aliquot of this; the total 24 hr. volume being also quoted so that the total 24 hr. excretion may be calculated. All patients included have a history of more than 2 years of cancer.

The treatment of patients with enzyme inhibitors

Patients have been treated with a number of inhibitors (supplied by Messrs. Kemball, Bishop and Co. Ltd., London, E.3) to decrease the β-glucuronidase activity in urine. The estimation of urinary β-glucuronidase involves a 3-fold dilution so that the apparent inhibition obtained is always less than the actual

effect in the urine. Patients were treated with solutions of the inhibitors flavoured with " fruit concentrates ".

(a) *Calcium gluconate.*—The average inhibition of urinary β-glucuronidase activity on treatment with 10 g. calcium gluconate is only 15 per cent (Table I).

TABLE I.—*Patients Suffering from Cancer of the Bladder Treated with Calcium Gluconate*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	M.	59	2750	1·8	2700	1·2
			2750	1·6	2500	0·8
2	„	63	1400	5·8	2750	3·8
			2350	5·5	2750	5·8
			4000	5·8	—	—
3	„	77	1800	1·7	2400	1·7
			2000	1·9	2850	1·8
			1950	2·0	2850	1·6
4	„	67	2750	1·5	3100	1·4
			3500	1·8	2700	1·3
			2750	1·2	3700	1·4
5	„	65	2900	1·9	3100	1·6
			3100	2·1	2500	1·3

(b) *Sodium ammonium saccharate.*—A patient with multiple tumours of the bladder was treated with sodium ammonium saccharate solution (10 g. per day for 1 week) (Fig. 2). The treatment was then stopped and the β-glucuronidase activity rose to the original value within 3 days. The variation in enzyme activity and the volume of the 24 hr. urine specimen is also recorded. The results obtained from 4 patients treated in a similar manner (Table II) indicate that the treatment reduced the β-glucuronidase activity by 31 per cent.

TABLE II.—*Patients Suffering from Cancer of the Bladder Treated with Sodium Ammonium Saccharate*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	M.	52	3700	3·2	3100	1·2
			4000	2·7	3500	1·7
			3100	2·6	3900	1·2
2	„	75	1700	3·0	3300	2·7
			2000	2·3	2800	2·0
			1800	2·3	—	—
3	„	62	2500	1·6	1900	1·1
			4300	1·3	2100	1·0
			2700	1·7	—	—
4	„	38	8000	0·8	3300	1·1
			6700	1·2	3600	0·7
			9250	1·2	3500	0·9
			7300	1·0	4400	1·9

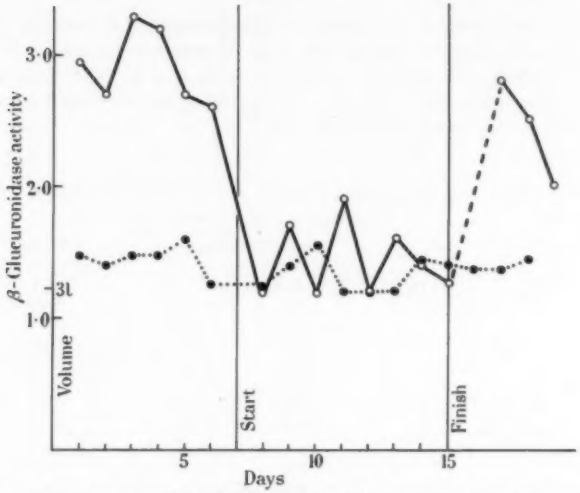


FIG. 2.—The effect of oral administration of sodium ammonium saccharate on urinary β-glucuronidase activity in a patient with bladder cancer.

○———○ β-Glucuronidase activity (units/ml.).
●·····● Urine volume (litres/24 hr.).

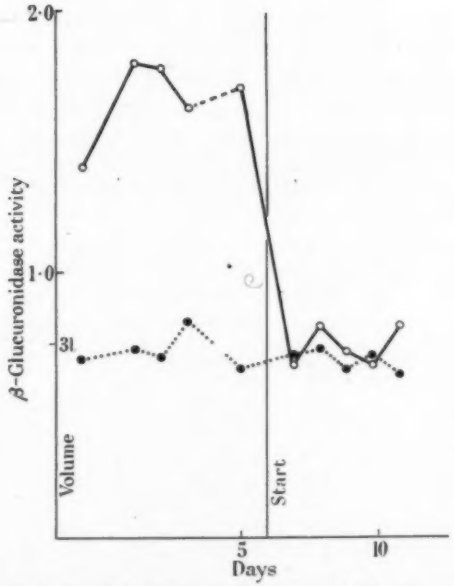


FIG. 3.—The effect of oral administration of ammonium hydrogen saccharate on urinary β-glucuronidase activity in a patient with bladder cancer.

○———○ β-Glucuronidase activity (units/ml.).
●———● Urine volume (litres/24 hr.).

(c) *Ammonium hydrogen saccharate.*—Treatment of a patient with 10 g. per day of ammonium hydrogen saccharate (Fig. 3) produced a fall in β-glucuronidase activity of the urine. Treating 6 patients with 3·3 g. of ammonium hydrogen saccharate 3 times daily produced an average apparent inhibition of the β-glucuronidase activity of 47 per cent (Table III).

TABLE III.—*Patients Suffering from Cancer of the Bladder Treated with Ammonium Hydrogen Saccharate*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	F.	51	2700	1·4	2750	0·65
			2850	1·8	2810	0·80
			2750	1·8	2520	0·70
			3250	1·6	2750	0·65
			2520	1·7	2450	0·80
2	M.	54	3100	0·50	2800	0·30
			2850	0·65	2700	0·35
3	„	47	3600	1·8	2000	1·2
			2500	1·4	2250	1·0
4	„	61	1800	1·6	2100	0·60
			1650	1·7	2200	0·65
			2750	1·6	2600	0·80
5	„	56	700	0·55	1600	1·0
			700	0·92	—	—
6	F.	56	800	2·6	2100	1·25
			2000	3·6	2600	1·1
			2000	1·9	—	—

(d) *Glucosaccharolactone.*—(1) A group of patients were treated with a boiled solution of saccharic acid which contains an equilibrium mixture of saccharic acid and 3 : 6 and 1 : 4 saccharolactones, and received 1·3 g. of saccharo-1 : 4-lactone 3 times daily. The effect of this treatment on the urinary β-glucuronidase activity (Fig. 4 and Table IV) indicates that the mean inhibition of β-glucuronidase activity is 92 per cent.

TABLE IV.—*Patients Suffering from Cancer of the Bladder Treated with Boiled Saccharic Acid Solution*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	M.	54	2000	0·90	2400	0·01
			1900	1·3	3100	0·03
			2600	1·4	3300	0·02
			1600	1·0	2900	0·00
2	F.	38	2500	1·4	2500	0·35
			2650	0·92	2610	0·21
			2520	1·6	2120	0·14
			2760	1·0	1800	0·07
3	„	55	2550	1·6	1100	0·44
			1750	1·0	2500	0·06
4	M.	48	980	3·6	1010	0·10
			910	2·7	1100	0·05
5	„	34	3060	1·22	4050	0·08
			4460	0·96	3700	0·10

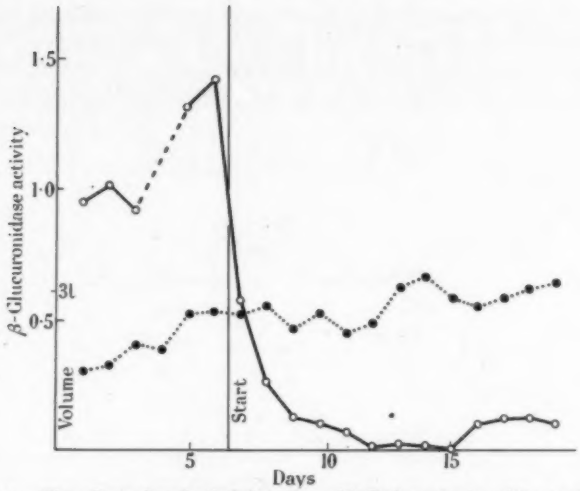


FIG. 4.—The effect of oral administration of boiled saccharic acid on urinary β-glucuronidase activity in a patient with bladder cancer.

○———○ β-Glucuronidase activity (units/ml.).
●·····● Urine volume (litres/24 hr.).

(2) Treatment of a number of patients with a mixture of glucosaccharo-1 : 4-lactone and glucosaccharo-3 : 6-lactone (2·5 g. of the mixture containing 1 g. of the 1 : 4-lactone administered 4 times daily) (Table V) caused an average inhibition of activity of 84 per cent.

TABLE V.—*Patients Suffering from Cancer of the Bladder Treated with a Mixture of Glucosaccharo-1 : 4-lactone and Glucosaccharo-3 : 6-lactone*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	F.	—	1825	4·7	2210	1·6
			1550	10·3	2140	1·2
2	„	76	1595	7·6	1865	1.3
			1535	6·1	—	—
			1335	6·1	—	—
3	M.	66	2640	6·3	1940	1·7
			2850	7·3	1725	0·28
			2550	6·2	1585	0·56
4	„	61	2700	2·8	2610	0·33
			2100	3·1	2490	0·33
5	„	82	1510	4·9	2100	0·71
			1575	3·2	1765	0·43

(3) The results obtained by treatment of patients with pure saccharo-1 : 4-lactone (1 g. 4 times daily) (Table VI) show a mean inhibition of 93 per cent. The effect of administration of both pure saccharo-1 : 4-lactone and of the 1 :4,

3 : 6 mixture on the urinary β-glucuronidase of patients suffering from cancer of the bladder is shown in Fig. 5. Specimens of urine were collected from each of 5 patients at approximately 3 hourly intervals and the β-glucuronidase activity of the individual specimens estimated. In 5 successive periods each of 48 hr. the

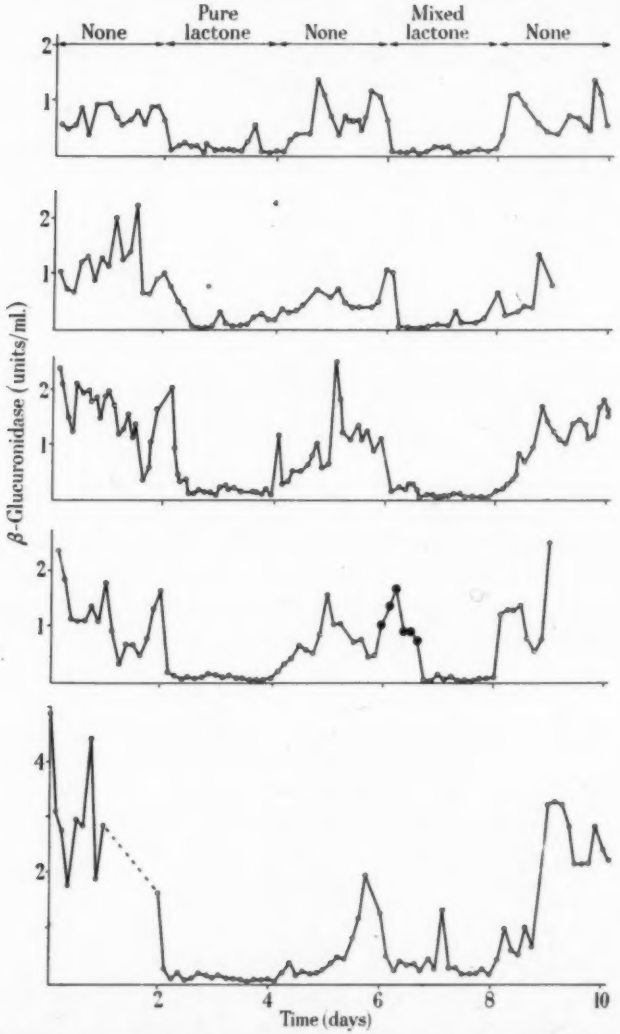
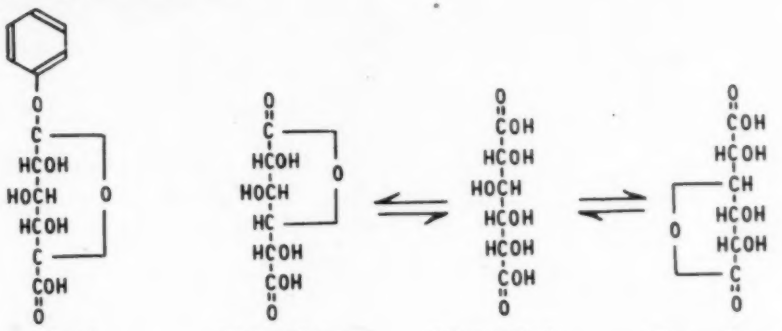


FIG. 5.—The effect of oral administration of glucosaccharo-1 : 4-lactone and mixtures of 1 : 4 and 3 : 6-lactones on urinary β-glucuronidase activity in 5 patients with bladder cancer.

○——○ β-Glucuronidase activity (units/ml.).
●——● β-Glucuronidase activity (units/ml.) in urine containing blood.

patients were given (1) no treatment, (2) treatment with pure saccharo-1 : 4-lactone (1 g. six hourly), (3) no treatment, (4) treatment with a mixture of saccharo-1 : 4- and 3 : 6-lactones/2 : 3 (2·5 g. 6 hourly), (5) no treatment. In Case 4 the urine was contaminated with blood on the seventh day which accounts for the high result obtained.



Phenyl Glucosiduronic acid (I) — Glucosaccharo-1 : 4-lactone (II) — Glucosaccharic acid (III) — Glucosaccharo-3 : 6-lactone (IV)

TABLE VI.—*Patients Suffering from Cancer of the Bladder Treated with Pure Glucosaccharo-1 : 4-lactone (1g. 4 times daily)*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
1	M.	53	1373	7·4	1527	0·84
			2013	5·2	1855	0·65
			1746	7·7	2120	0·51
2	„	62	2050	7·3	2105	0·88
			1760	6·3	2110	0·22
3	F.	76	2040	10·1	1980	0·60
			1660	7·7	1565	0·90
			2125	7·1	—	—
4	M.	66	2315	5·9	2110	0·88
			2740	6·6	—	—
5	F.	—	1470	12·6	2190	0·64
			1920	11·2	1695	0·44
6	M.	61	1980	3·0	2120	0·48
			2850	2·8	1870	0·32
7	„	82	1720	5·5	1560	0·22
			1600	4·1	1590	0·37

(4) The results obtained by treating patients with pure saccharo-1 : 4-lactone (0·5 g. 3 or 4 times daily) (Table VII) show a mean inhibition of 73 per cent and the effect on individual urine specimens of treating patients with 0·5 and 1 g. of saccharo-1 : 4-lactone 8 hourly is shown in Fig. 6.

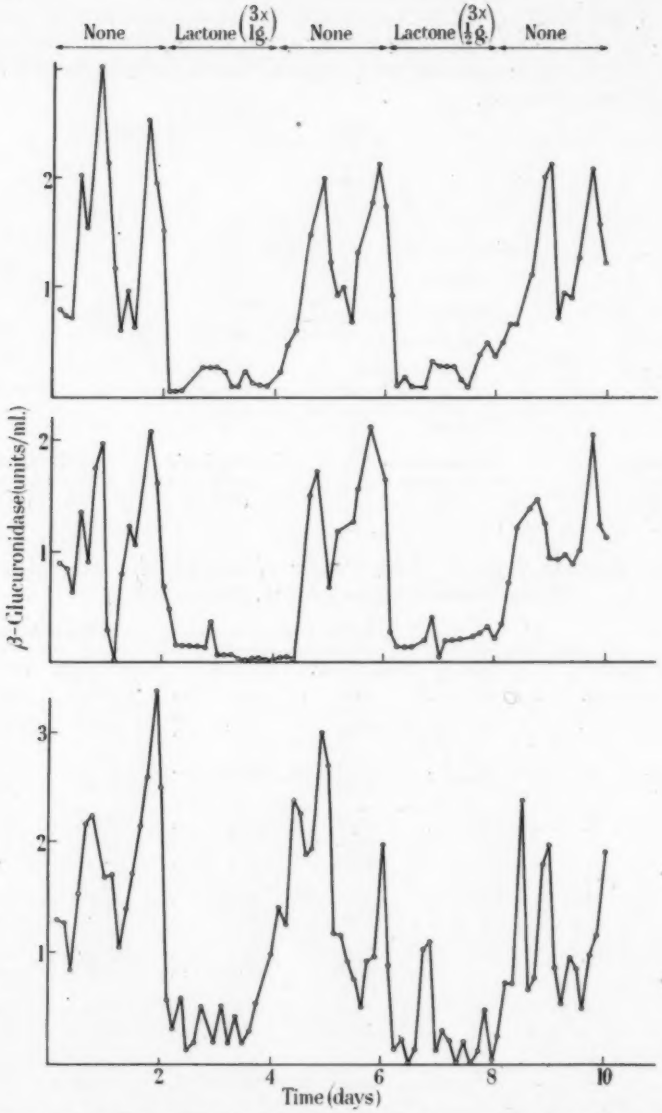


FIG. 6.—The effect of oral administration of pure glucosaccharo-1 : 4-lactone on urinary β-glucuronidase activity in 3 patients with bladder cancer.
○——○ β-Glucuronidase activity (units/ml.).

TABLE VII.—*Patients Suffering from Cancer of the Bladder Treated with Pure Glucosaccharo-1 : 4-lactone*

Case No.	Sex	Age	Before treatment		During treatment	
			Vol./24 hr.	Units/ml.	Vol./24 hr.	Units/ml.
(*a*) Dose 4 × 0·5 g./day)—						
1	M.	52	2720	1·1	2780	0·12
			2585	0·83	2740	0·12
2	„	63	1415	1·5	1205	0·16
			1600	4·4	—	—
3	F.	43	1100	0·53	1550	0·06
			1700	0·38	1835	0·13
(*b*) (Dose 3 × 0·5 g./day)—						
1	M.	43	1330	6·5	1515	3·8
			1205	6·9	1405	3·1
2	„	55	2350	1·4	2140	0·17
			1560	1·5	2310	0·25
3	„	68	2800	0·93	1830	0·10
			1590	1·0	2060	0·22
4	„	71	2360	1·7	2290	0·37
			1510	1·9	2750	0·47

The results indicate that the urinary β-glucuronidase activity can be maintained at a low level by the administration of either the pure 1 : 4-lactone or the mixture of lactones. Patients are being treated with 1 g. saccharo-1 : 4-lactone *t.d.s.* for clinical trials.

DISCUSSION

The urinary β-glucuronidase activities of patients who have been suffering from cancer of the bladder for some years are almost all raised above the corresponding value for normal subjects. The increased activity does not depend upon infection although, in a few cases infected by *B. coli*, this may play some part. The enzyme is stable up to pH 9·0 and the method of estimation allows urine of pH less than this value to be used. The results are in agreement with those obtained previously (Boyland *et al.*, 1955) using an earlier technique.

The urinary β-glucuronidase activity of patients with cancer of the kidney (hypernephroma) is low compared with that of bladder cancer patients, the average value being within the normal range. Fishman and his co-workers have shown that cancer cells have a high β-glucuronidase activity but the high urinary activity in cancer of the bladder cannot be due to the urine being in contact with malignant tissue as urine from patients with cancer of the kidney should also be rich in β-glucuronidase. This evidence further supports the suggestion (Boyland *et al.*, 1955) that the increased urinary enzyme concentration is probably derived from the blood, the amount of the enzyme depending both on the blood concentration and the degree to which the enzyme passes through the kidney. The relationship between the β-glucuronidase activities of serum and urine of patients with cancer of varying sites is being investigated further.

Patients suffering from cancer of the bladder have been treated with glucosaccharo-1 : 4-lactone (4 g. per day) which reduces the activity by 90 per cent. This inhibition of β-glucuronidase would be produced with a 5×10^{-5} M saccharo-

1 : 4-lactone solution (Levvy, 1952) which is equivalent to about 10 mg. per litre. This indicates that only 0·5 per cent of the dose administered is excreted in the urine. The greater part (99·5 per cent) of the lactone is inactivated either by metabolic processes or reaction with water or other body constituents. This treatment reduced the urinary β-glucuronidase level to values below the average normal level and by this means it is hoped to decrease the amount of free carcinogens in the urine and thus reduce the incidence of new tumours in patients, who have had tumours removed. Twenty bladder cancer patients are at present under treatment with glucosaccharo-1 : 4-lactone, but this treatment has not been used long enough to allow any conclusions to be drawn as to the clinical benefit.

The authors realise that to treat patients who have had cancer of the bladder with compounds which will reduce the concentration of carcinogenic agents in the urine, is probably to act too late. Because of the long lag between the application of the carcinogenic stimulus and the occurrence of the cancer, the first stimulus had probably taken effect before treatment began. This is, however, not certain and the reduction of carcinogens in the urine may reduce the chance of tumours developing. The tumours may, however, be dependent on the continued presence of the carcinogen in the way that the growth of kidney tumours, induced in hamsters with stilboestrol is dependent on the continued presence of stilboestrol (Horning, 1956). If the tumours of the bladder were dependent on the urinary carcinogen then treatment with saccharo-1 : 4-lactone could prevent growth of established tumours.

Bladder cancer caused by aromatic amines among men in the chemical industry is probably due to metabolites of the aromatic amines being hydrolysed by β-glucuronidase in the urine to give aminophenols. Stringent precautions are now taken in the chemical industry to prevent exposure to the carcinogenic amines. In such factories, however, if accidents happen the carcinogenic effect of exposure of personnel should be reduced by administration of saccharo-1 : 4-lactone immediately following the exposure and for four or five days. In such cases the treatment should prevent the liberation of the carcinogen in the urine.

SUMMARY

The *in vivo* effect of various derivatives of saccharic acid which are β-glucuronidase inhibitors has been investigated ; oral administration of 1 g. saccharo-1 : 4-lactone every 6 hours decreases the enzyme activity in the urine by 90 per cent.

Treatment with saccharolactone should therefore reduce the liberation of free carcinogenic aminophenols in urine. Such treatment is recommended for men accidentally exposed to carcinogenic aromatic amines and is being tried in prophylactic treatment of patients who have had bladder cancers removed or destroyed.

We are indebted to Mr. L. M. Miall of Messrs. Kemball Bishop and Co. Ltd., for his help in supplying many of the substances used in this work.

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Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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SOME PROPERTIES OF THE MITOCHONDRIA OF TUMOUR CELLS

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IN a previous paper (Abrignani and Mutolo, 1957) it has been shown that mitochondria prepared from tumour cells when incubated with versene lose a greater amount of compounds absorbing maximally at 260 mμ than mitochondria from normal liver cells do. This observation has led us to suggest a possible lability of the membrane of tumour cell mitochondria. A similar suggestion has been recently presented by Emmelot and Brombacher (1957).

In the experiments to be reported in this paper the swelling properties of the mitochondria from tumour cells have been compared to those of mitochondria of normal liver cells. The differential response of the two kinds of mitochondria to detergents and trypsin has also been investigated.

The rationale behind these experiments was that should the membrane of tumour mitochondria be altered in its structure or chemical composition, this would probably reflect in a different response with respect to mitochondria from normal cells, to certain experimental conditions, as e.g. the lowering of the osmolarity of the suspending medium.

The results we have obtained support the view that such an alteration does in fact exist in the tumour mitochondria.

MATERIAL AND METHODS

Preparation of mitochondrial suspensions

Normal albino rats and rats bearing subcutaneous 256 Walker tumour were used in these experiments. The liver of normal rats and the solid tumours free of necrotic tissues were collected immediately after killing the animals and homogenized at 0° C. in a glass homogenizer with a teflon plunger. The homogenization was carried out in nine volumes of 0·44 M sucrose in 0·1 M citrate buffer pH 6·3 containing 10^{-3} M versene (ethylendiaminetetracetic acid, tetrasodium salt) according to Witter, Watson and Cottone (1955). Nuclei and cell debris were sedimented at 700 × g. for 10 minutes in the cold. The supernatant was then centrifuged at 10,000 × g. for 20 minutes (in the MSE refrigerated centrifuge) and the sedimented mitochondrial pellet was suspended in five volumes of 0·44 M sucrose in 0·1 citrate buffer pH 6·3 and centrifuged again at 24,000 × g. for 20 minutes. The final sediment was suspended in 0·5 M sucrose in 0·02 M tris(hydroxymethyl) aminomethane buffer at pH 7·4 making use of a homogenizer. For the suspension of liver mitochondria five volumes of sucrose were used whereas half this volume was used for the suspension of tumour mitochondria. The mitochondria were used immediately after preparation.

Experimental procedure

In the experiments to investigate the swelling of the mitochondria, 0·1 ml. of the suspension were added to 3·0 ml. of sucrose solutions ranging from 0·5 to 0·1 M (in 0·02 M tris buffer pH 7·4) and finally to 3·0 ml. buffer alone; the suspensions were kept at the room temperature (19–20° C.) for the duration of the experiments. Optical densities were read at 520 mμ in a Beckman Spectrofotometer starting 30 seconds after mixing and then after 2, 5, 10, and 15 minutes.

In the experiments with versene, this was used at the final concentration of $5 \cdot 10^{-4}$ M.

For the study of the effect of detergents 0·1 ml. of the original mitochondria suspension were diluted in 3·1 ml. either of 0·3 M sucrose or of tris buffer containing 83 μg. of Duponol or 0·86 μmoles of sodium deoxycholate.

In the experiments with trypsin, 150 μg. of crystalline (Armour) trypsin were added to 3·2 ml. of the mitochondria suspension in 0·3 M sucrose or in tris buffer.

No agglutination was ever observed in any one of the experiments.

RESULTS

Swelling experiments

These experiments have shown that the swelling properties of tumour mitochondria in hypotonic solutions are considerably different from those of liver mitochondria.

Fig. 1 indicates that liver mitochondria begin to swell at 0·3 M sucrose. By further lowering the sucrose concentration a parallel increase of the rate of swelling of the mitochondria takes place, as indicated by the rapid decrease of the optical density of the suspension at 520 mμ. The results are in good agreement with those of Tapley (1956).

Fig. 2 shows the changes in optical density of suspensions of tumour mitochondria at different concentrations of sucrose. It appears clearly that under these conditions the swelling of tumour mitochondria is considerably less than that of liver mitochondria.

These experiments indicate that tumour mitochondria maintain a low water content in spite of strongly hypotonic conditions of the medium.

Effect of versene on the swelling of mitochondria

Our experiments confirm that a $5 \cdot 10^{-4}$ M final concentration of versene effectively counteracts the swelling of liver mitochondria in hypotonic solutions.

On the contrary, as indicated in Fig. 3, the same concentration of versene appears to promote the swelling of tumour mitochondria.

Effect of detergents

As can be seen in Fig. 4, in the presence of both Duponol and sodium deoxycholate the decrease of the optical density of a mitochondria suspension is considerably greater in the case of liver than on tumour mitochondria.

Effect of trypsin

As reported by Dianzani (1953) a 0·04 per cent solution of crystalline trypsin is able to cause considerable damage to isolated liver mitochondria. In our

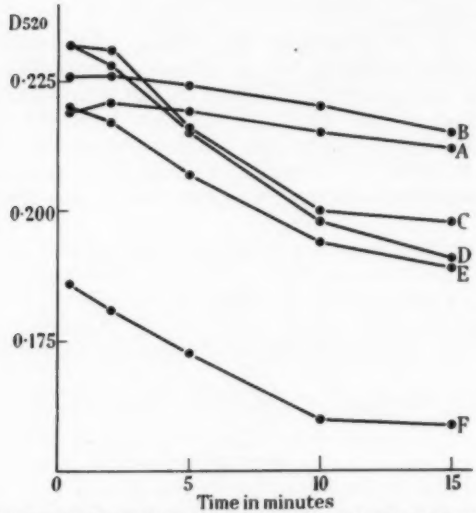

FIG. 1.—Swelling of liver mitochrondria. Change in optical density (at 520 mμ) of a suspension of liver mitochondria in (A) 0·5 M sucrose; in (B) 0·4 M sucrose; in (C) 0·3 M sucrose; in (D) 0·2 M sucrose; in (E) 0·1 M sucrose; in (F) 0·02 M tris buffer pH 7·4. Sucrose in 0·02 M tris buffer pH 7·4. Total volume of mixture was 3·1 ml. T = 19–20° C.

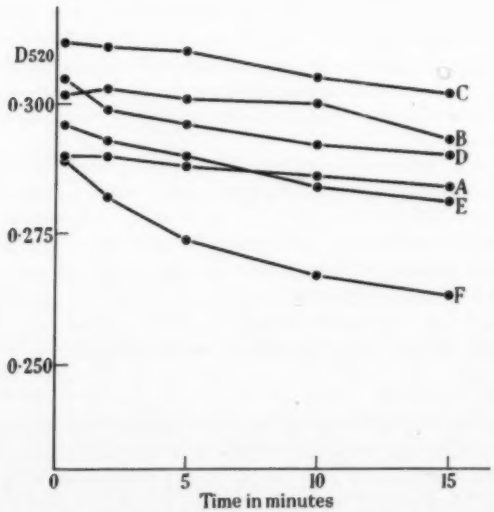

FIG. 2.—Swelling of tumour mitochondria. Change in optical density (at 520 mμ) of a suspension of tumour mitochondria in (A) 0·5 M sucrose; in (B) 0·4 M sucrose; in (C) 0·3 M sucrose; in (D) 0·2 M sucrose; in (E) 0·1 M sucrose; in (F) 0·02 M tris buffer pH 7·4. Sucrose in 0·02 M tris buffer pH 7·4. Total volume of mixture was 3·1 ml. T = 19–20° C.

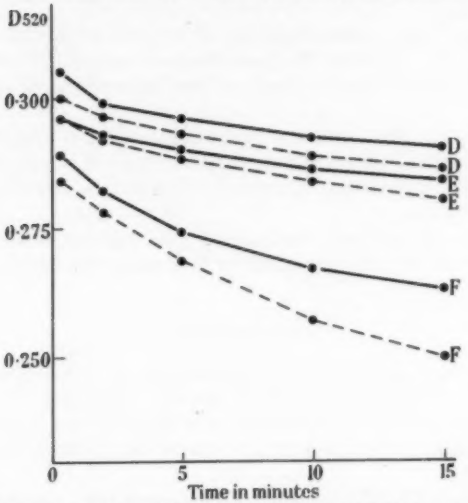

FIG. 3.—Effect of versene on the swelling of tumour mitochondria. Solid lines show the swelling of mitochondria in (D) 0·2 M sucrose (in 0·02 M tris buffer pH 7·4); in (E) 0·1 M sucrose (in 0·02 M tris buffer pH 7·4); in (F) 0·02 M tris buffer pH 7·4; broken lines with addition of $5 \cdot 10^{-4}$ M versene. Total volume of mixture was 3·1 ml. T = 19–20° C.

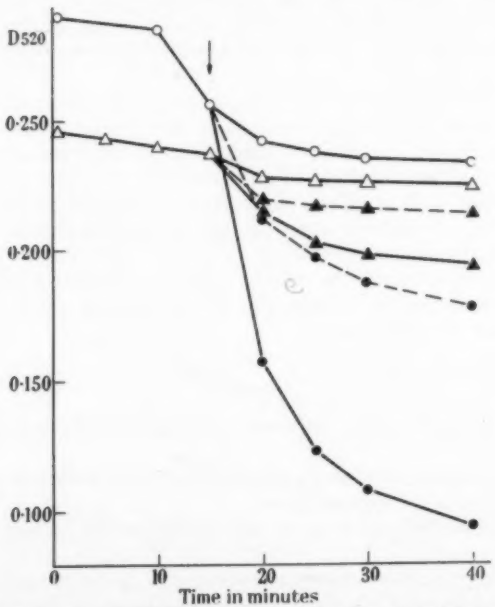

FIG. 4.—Effect of detergents. Control liver mitochondria (○—○) in 0·3 M sucrose (in 0·02 M tris buffer pH 7·4); the same with addition of 83 μg. of Duponol (●—●); and with 0·86 μmoles of sodium deoxicholate (●---●). Control tumour mitochrondria (△—△) in 0·3 M sucrose (in 0·02 M tris buffer pH 7·4); the same with addition of 83 μg. of Duponol (▲—▲); and with 0·86 μmoles of sodium deoxicholate (▲---▲). Detergents were added after 15 min. of preincubation. Final volume of mixture was 3·2 ml. T = 19–20° C.

experiments we have used concentrations of trypsin somewhat lower than those used by Dianzani as it is conceivable that relatively small differences in sensitivity to trypsin would be detected more likely at low rather than at high concentrations of the enzyme.

As shown in Fig. 5, suspensions of liver mitochondria incubated with trypsin in 0·3 M sucrose or in tris buffer show a slight increase of the optical density during the first 30 minutes ; a moderate lowering of the optical density occurs only after 60 minutes of incubation.

On the contrary, a marked decrease of the optical density takes place in suspensions of tumour mitochondria under the same conditions (Fig. 6).

DISCUSSION

Thus far the investigation of the properties of tumour mitochondria have revealed a number of differences with respect to mitochondria from normal cells in regard to their morphology (Howatson and Ham, 1955), protein and enzymatic composition (Hogeboom and Schneider, 1951 ; Schneider, Hogeboom, Shelton and Striebich, 1953).

The findings reported in the present paper suggest the existence of structural and chemical differences between the mitochondria of a tumour and those of the liver. The swelling properties of the mitochondria under passive experimental conditions, i.e. in which presumably no metabolic activity is occurring, indicate that tumour mitochondria have a much more limited permeability to water.

A structural-chemical difference between the proteins of the membrane of the two kinds of mitochondria is further indicated by the results of the experiments with detergents and trypsin.

The fact that the proteins of the tumour are more susceptible to trypsin may indeed be taken as an indication either that lipids play a less important role in the structure of such mitochondria or that their proteins are in a condition which one would indicate as denaturated. The former interpretation seems to be substantiated by the much weaker effect which anionic detergents, known to be very active on lipoprotein structures, exert on tumour mitochondria.

The present authors are well aware that in order for these results to gain general value and significance the mitochondria of several kinds of tumours and of different organs should be examined. Investigations along these lines are, in fact, in progress at this laboratory.

SUMMARY

Differences have been found between mitochondria from normal rat liver and Walker tumour.

The tumour mitochondria suspended in hypotonic solution have shown much less swelling than those of normal liver.

Tumour mitochondria prove to be susceptible to the action of detergents and to the effect of trypsin.

These results suggest that changes occur in mitochondria membrane of tumour.

We wish to thank Prof. A. Monroy for valuable advice and helpful discussions.

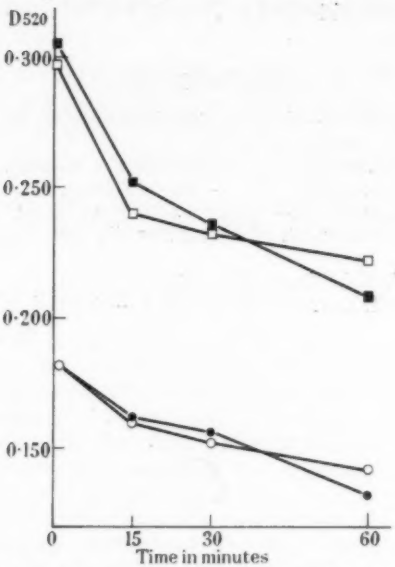

FIG. 5.—Effect of trypsin on liver mitochondria. Mitochondria were suspended in 0·3 M sucrose (in 0·02 M tris buffer pH 7·4) without trypsin (□—□) and with 150 μg. of trypsin (■—■); in 0·02 M tris buffer pH 7·4 without trypsin (○—○) and with 150 μg. of trypsin (●—●). Total volume of mixture was 3·2 ml. T = 19–20° C.

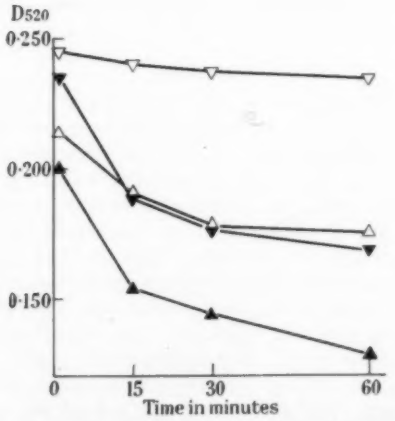

FIG. 6.—Effect of trypsin on tumour mitochondria. Mitochondria were suspended in 0·3 M sucrose (in 0·02 M tris buffer pH 7·4) without trypsin (▽—▽) and with 150 μg. of trypsin (▼—▼); in 0·02 M tris buffer pH 7·4 without trypsin (△—△) and with 150 μg. of trypsin (▲—▲). Total volume of mixture was 3·2 ml. T = 19–20° C.

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STUDIES ON CERTAIN PROTEINS IN NORMAL AND PATHOLOGICAL EPIDERMIS

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THE manner by which carcinogenic agents induce neoplastic growths is unknown in spite of the large amount of work which has been carried out on the chemistry of carcinogenesis. Epidermis was chosen as the tissue of choice for the work reported here for reasons given previously (Carruthers, 1950). Various stages in the malignant process such as methylcholanthrene-induced hyperplastic epidermis, papillomas and squamous-cell carcinomas were investigated. These phases were studied because large changes were sought in the present study particularly since the various degrees of intermediate and late epidermal hyperplasia show little, if any, significant chemical alterations (Carruthers, 1950). Also the recent studies of Rudall (1952) on the structural (urea extractable) proteins of beef snout epidermis offer some background for the proposed work. The structural proteins of epidermis may play a role in the adhesiveness of squamous cells (Rudall, 1952) and alterations in such proteins may account for the invasiveness and metastasis of skin carcinomas. In addition to the urea extractable protein (Carruthers, Woernley, Baumler and Kress, 1955) physical chemical studies were carried out on an alkali soluble protein of epidermis (Carruthers, Woernley, Baumler and Shorts, 1955 ; Woernley, Carruthers, Regent and Baumler, 1957).

METHODS

The hair from groups 70 to 100 of Bagg or Swiss albino mice was removed from the back with an electric clipper prior to the application of methylcholanthrene (0·6 per cent in benzene) or of croton oil (1 per cent in the same solvent) with a camel-hair brush No. 4 to an area about 2·5 cm. wide and 4·5 cm. long. Croton oil treated epidermis served as a control since a huge number of normal mice would have to be sacrificed to yield sufficient epidermis from which to extract the proteins in large enough quantities for characterization. Croton oil treated epidermis shows a relatively benign hyperplasia since numerous applications of this material are necessary to elicit a good carcinogenic response in many mice (Roe, 1956). The epidermis was removed from the dermis at 50° C. (Baumberger, Suntzeff and Cowdry, 1942). Papillomas from many mice were pooled and then stored at 0–4° in 95 per cent alcohol until the amount was ample for extraction of the various proteins. Methylcholanthrene induced squamous-cell carcinomas were pooled from some 15 to 20 mice, and a small piece from each cancer was fixed and stained for histological examination. The remainder was cleaned of necrotic and connective tissue and placed in 95 per cent alcohol. The urea extract-

able and alkali soluble proteins were isolated and purified as previously described (Woernley, Carruthers, Regent and Baumler, 1957; Carruthers, Woernley and Hittleman, 1957).

In the sedimentation measurements a synthetic boundary cell was used with a Spinco Model An-D analytical rotor in a Model E ultracentrifuge. The advantage of this type of cell is that sharp schlieren peaks are initially formed for the more or less polydisperse proteins under investigation (Woernley, Carruthers, Regent and Baumler, 1957). Sedimentation constants, s_t, were calculated from the slopes of log x versus t plots where x is the distance in cm. of the schlieren peak from the axis of rotation at time t in seconds. The s_{20} values were obtained with the aid of Svedberg's reduction formula (Svedberg and Pedersen, 1940). The centrifugal force employed was 259,700 $\times$ g.

Viscosities were determined on an Ostwald-Fenske viscometer with a flow time for water of 245·5 sec. at 20° C. All samples for viscometry were subjected to centrifugation to remove sources of interference to capillary flow such as foreign particles. Mobilities were measured in the usual manner in a Perkin-Elmer Model 208 electrophoresis apparatus (Moore and White, 1948; Moore, Roberts, Costello and Schonberger, 1949).

RESULTS

In Rudall's (1952) original studies on the fibrous and non-fibrous (structural) proteins of beef snout epidermis, maximum insolubility of the former at pH 5·5 and of the latter at pH 4·5 respectively was employed for isolation and purification procedures. The mobility of each of these proteins was determined in 0·2 ionic strength buffers (Miller and Golder, 1950) as a function of pH. Due to the insolubility of these proteins between pH 4 and 6, accurate measurement of the isolectric points was impossible. Fig. 1 and 2 show respectively the variation in mobility with pH of the fibrous and non-fibrous proteins of beef snout epidermis. In the electrophoresis of these protein preparations a single electrophoretic component was usually present; these patterns were quite sharp at the lower pH levels but became broader above pH 7·0. These observations indicate that the two proteins are fairly homogeneous.

Homogeneity is further indicated in the electrophoretic patterns of the alkali soluble protein (Fig. 3) and of the urea extractable protein (Fig. 4) of methylcholanthrene induced papillomas. These patterns were thus employed for the calculations of the mobility of the urea and alkali soluble proteins of normal and pathological epidermis.

The mobility of the urea extractable protein of the squamous cell carcinomas was somewhat greater than that of the protein of papillomas and of croton oil oil and methylcholanthrene treated epidermis (Table I). The average mobility of the alkali soluble protein (which precipitated maximally at pH 5·5 and hereafter denoted as the alkali soluble protein (Woernley, Carruthers, Regent and Baumler, 1957)) was nearly the same for the carcinomas, papillomas and treated epidermis. The average mobility of the urea and alkali soluble protein of human epidermis was greater than that of croton oil or methylcholanthrene treated mouse epidermis (Table I). Extraction of two samples of human basal cell carcinomas with 6M urea failed to yield any protein which precipitated at pH 5·5 although the one which flocculated at pH 4·5 was obtained in the usual manner.

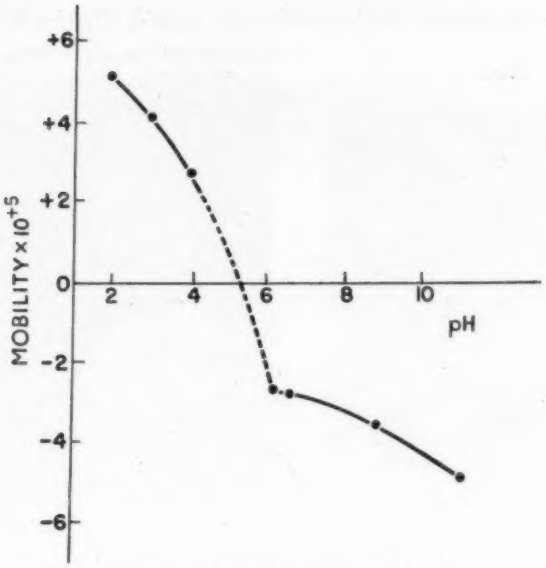

FIG. 1.—pH-mobility curve of the fibrous protein of beef snout epidermis.

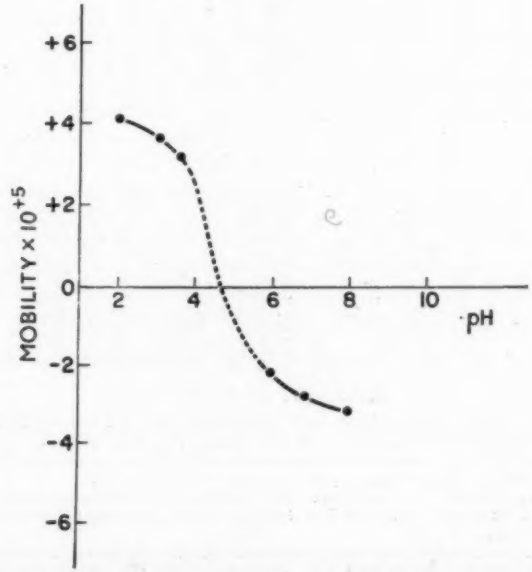

FIG. 2.—pH-mobility curve of the non-fibrous protein of beef snout epidermis.

TABLE I.—*Mobility of the Urea and Alkali Soluble Proteins of Epidermis*

Tissue	Urea extractable protein mobility† $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^5$	Alkali soluble protein mobility† $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^5$
Croton oil treated epidermis	2·3±0·3 (5)*	3·4±0·3 (4)
Methylcholanthrene treated epidermis	2·6±0·4 (4)	3·0±0·3 (3)
Papillomas	2·7±0·1 (2)	3·1 (1)
Squamous-cell carcinomas	3·0±0·3 (4)	2·9±0·1 (3)
Human epidermis	3·8±0·6 (5)	4·2±0·1 (3)
Beef snout epidermis	2·8±0·2 (5)	3·4±0·2 (3)

* Numbers of samples are given in parentheses. For the croton oil and methylcholanthrene treated groups 70 to 100 mice were used for each analysis and for the papillomas and carcinomas, about 300 and 20 mice respectively were employed.

† Electrophoresis was carried out in phosphate buffer—NaCl solution of pH 7·4 ($\Gamma/2$, 0·2) at 1·5° C.

The mobility of the proteins from beef snout epidermis was quite similar to that obtained for the proteins from mouse epidermis. The mobility of the urea and alkali soluble proteins of beef snout epidermis was not appreciably affected by pre-extraction of this tissue with a solution of ether and alcohol (equal volumes) or with ether alone. These experiments were carried out since the lipids of mouse and human epidermis had to be removed prior to the extraction of this tissue with 6M urea. This step was necessary since urea-lipid complexes, which formed creamy homogenates, prevented isolation of the various proteins.

The sedimentation constants, s_t, were determined at 3–5 different concentrations of the proteins in 0·05M borate buffer and in 0·05M borate in 6M urea. A plot of log x versus t where x is the distance in cm. of the schlieren peak from the axis of rotation at time, t, in seconds, is shown in Fig. 5. The s_{20} values were then obtained with the aid of Svedberg's reduction formula.

The average s_{20} values and intrinsic viscosity $[N]$, of the urea and alkali-soluble proteins of mouse epidermis in various states are shown in Table II. There was no significant change in the average s_{20} value of either protein in the epidermis undergoing carcinogenesis. The s_{20} value of the urea extractable protein was nearly the same in borate buffer as in borate buffer-6M urea. However, the schlieren peaks of this protein were much sharper in borate-6M urea solution (Fig. 6) than they were in the borate buffer alone (Fig. 7). The s_{20} constants of the alkali soluble proteins were larger than those of the urea extractable protein and they showed some decrease in borate-6M urea solution as compared to borate buffer alone.

There was no significant difference in the average intrinsic viscosity of the urea soluble protein of epidermis undergoing carcinogenesis (Table II) and the values

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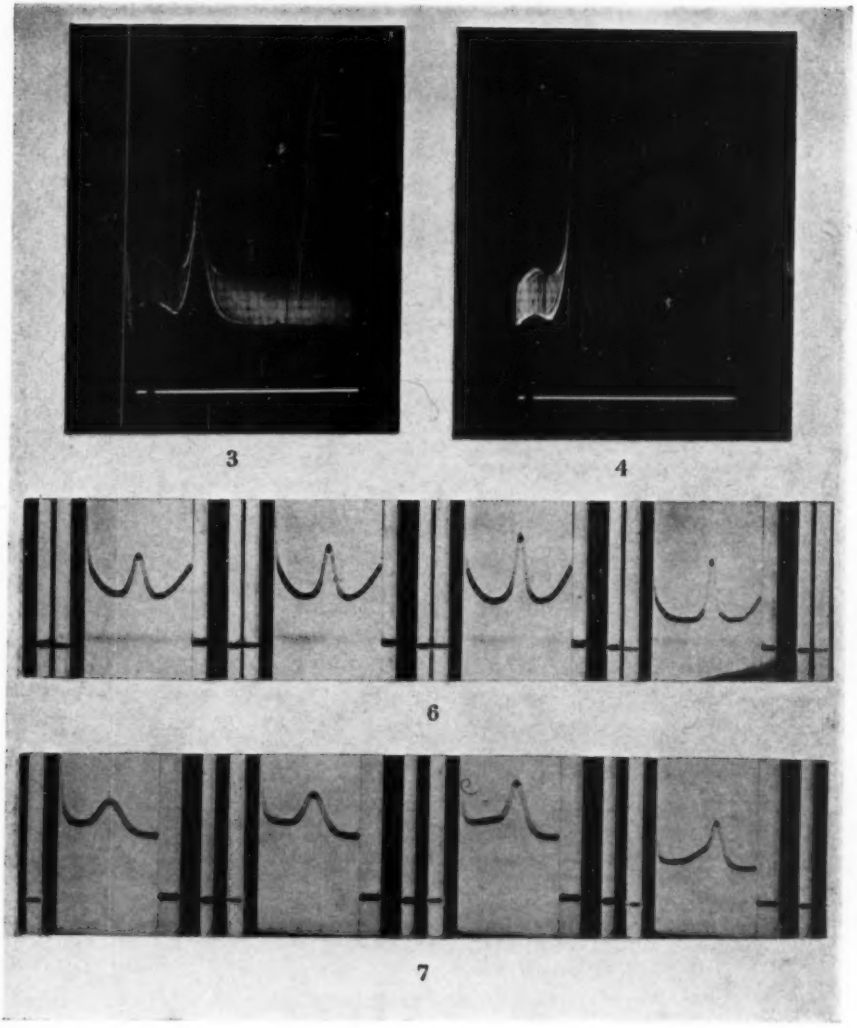
EXLANATION OF PLATE.

FIG. 3.—Electrophoretic pattern of the urea extractable protein from papillomas descending limb, in phosphate buffer-NaCl solution of pH 7·4 ($\Gamma/2$, 0·2) at 1·5° C.

FIG. 4.—Electrophoretic pattern of the alkali soluble protein from papillomas descending limb, in phosphate buffer-NaCl solution of pH 7·4 ($\Gamma/2$, 0·2) at 1·5° C.

FIG. 6.—Schlieren patterns for urea extractable protein of squamous-cell carcinomas; 0·05M borate buffer in 6M urea; concentration 0·66 g./100 ml. solution; time interval between photographs, 8 min.

FIG. 7.—Schieren patterns for urea extractable protein of squamous-cell carcinomas; 0·05M buffer; concentration 1·08 g./100 ml. solution; time interval between photographs, 8 min.



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6

7

Carruthers, Woernley, Baumler and Lilga.

were nearly the same in borate buffer as in borate buffer-6M urea solution. The average intrinsic viscosity of the alkali soluble protein of the carcinomas in borate buffer was less than that of the treated epidermis. The average intrinsic viscosity of this protein in borate buffer-6M urea solution was nearly twice that found in the borate buffer alone for each tissue. The decrease in the intrinsic viscosity in order from croton oil treated and methylcholanthrene treated epidermis, papillomas

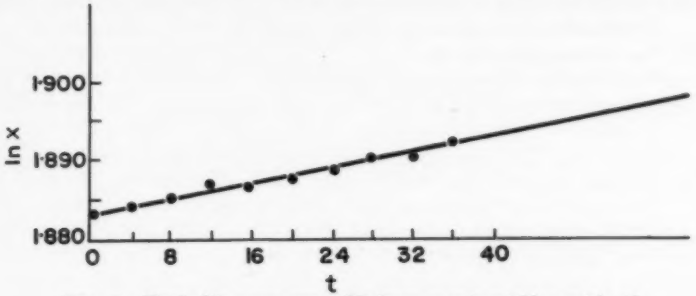

FIG. 5.—Typical log x versus t plot for urea extractable protein of croton oil treated epidermis.

TABLE II.—*Sedimentation Constant and Intrinsic Viscosity of the Urea and Alkali Soluble Proteins of Mouse Epidermis Undergoing Carcinogenesis*

	Urea extractable protein in				Alkali soluble protein in			
	0·05M borate†		0·05M borate† in 6M urea		0·05M borate†		0·05M borate† in 6M urea	
Tissue	s_{20}	$[N]$	s_{20}	$[N]$	s_{20}	$[N]$	s_{20}	$[N]$
Croton oil treated epidermis	1·8±0·2(3)*	0·16±0·03(3)	1·9±0·1(2)	0·26±0·06(3)	3·0±0·3(3)	0·48±0·09(6)	2·4±0·2(2)	1·1±0·3(6)
Methylcholanthrene treated epidermis	1·8±0·2(3)	0·21±0·04(2)	1·7±0·1(3)	0·27±0·01(3)	3·3±0·6(2)	0·49±0·09(3))	2·2±0·1(2)	0·9±0·1(3)
Papillomas	1·9±0·2(3)	0·11±0·03(2)	1·9	(1) 0·33±0·07(2)	3·5	(1) 0·43±0·10(2)	— —	0·8 (1)
Squamous cell carcinomas	1·9±0·2(3)	0·21±0·03(2)	1·9±0·1(2)	0·23±0·03(3)	2·6±0·5(3)	0·27±0·09(5)	2·2±0·1(2)	0·6±0·3(5)

* Numbers of samples are given in parentheses. Number of mice used per analysis is the same as given in Table I.

† 0·05 moles $Na_2B_4O_7 10H_2O$ dissolved in a liter of distilled water.

and carcinomas may be of some significance but more analysis would be necessary to prove this. The increase in intrinsic viscosity of the alkali soluble protein in borate buffer urea solution as compared to that in borate buffer alone is probably due to an increase in axial ratio or asymmetry of the protein molecule ((Putnam, 1953).

The s_{20} and intrinsic viscosity of the urea and alkali soluble proteins of human and beef snout epidermis are shown in Table III. The s_{20} and intrinsic viscosity

values were of the same order of magnitude for the proteins of each type of epidermis and they were greater than for the same proteins of croton oil treated mouse epidermis. The decrease in s_{20} of the alkali soluble proteins in borate-6M urea buffer as compared to the buffer alone was similar to that found for mouse epidermis.

TABLE III.—*Sedimentation Constant and Intrinsic Viscosity of the Urea and Alkali Soluble Proteins of Human and Beef Snout Epidermis*

Type of epidermis	Urea extractable protein in 0·05M borate†		Urea extractable protein in 0·05M borate† in 6M urea		Alkali soluble protein in 0·05M borate†		Alkali soluble protein in 0·05M borate† in 6M urea *f*	
	s_{20}	$[N]$	s_{20}	$[N]$	s_{20}	$[N]$	s_{20}	$[N]$
Human	2·3±0·1(4)*	0·27±0·07(4)	1·6±0·1(3)	0·48±0·11(3)	4·2±0·1(3)	0·73±0·13(4)	2·4±0·1(3)	0·91±0·19
Beef snout	2·1±0·3(4)	0·28±0·07(5)	1·8 (1)	0·30±0·09(3)	4·3 (1)	0·54 (1)	2·3 (1)	0·67

* Numbers of samples are given in parentheses.
† 0·05 moles $Na_2B_4O_7.10H_2O$ dissolved in a liter of distilled water.

The partial specific volume V of the urea extractable protein from croton oil treated epidermis was determined with borate buffer as the solvent, and assuming no solvation a value, $V = 0{\cdot}744$ c.c./g. was calculated from density measurements. In Table IV are listed viscosity increments, extrapolated to zero volume fraction ($(\varphi = 0)$, axial ratios, frictional coefficients, s_0 values and average molecular weights of the urea and alkali extractable proteins in 0·05M borate buffer. In the calculation for axial ratio frictional coefficient and molecular weight a prolate ellipsoid-shaped molecule was assumed (Golder, 1953; Simha, 1940).

From Table IV, it is seen that the urea extractable protein has a lower molecular weight than the protein extracted by alkali from the residue of the urea extracted epidermis. The latter in its original state is probably held together by stronger bonds than the former and the alkali soluble protein is probably more keratinous.

TABLE IV.—*Viscosity Increments, Axial Ratios, Frictional Coefficients, Sedimentation Constants and Molecular Weights of Proteins of Croton Oil Treated Mouse Epidermis in the Solvent,* 0·05M *Borate Buffer*

Extractant	Viscosity increment ($\varphi = 0$)	Axial ratio	Frictional coefficient	s_0 Sveds	Molecular weight
6M urea	21·5	13·8	1·7	1·8	28,000
0·05N NaOH	64·5	27·5	2·3	3·0	91,000

DISCUSSION

The investigations of many workers have indicated that the adhesiveness of squamous cells is probably due to the nature of the proteins associated with intracellular bridges or tonofibrils (Giroud and Leblond, 1951; Mercer, 1949). Rudall's studies on the fibrous protein of epidermis indicated that this protein, a prekeratin, is a part of the tonofibril (Rudall, 1952). Hence, it appeared reasonable to assume that this protein might be of some importance in the carcinogenesis

of epidermis. This is so because carcinomas of this tissue in man metastasize, a process requiring the freeing of cells or groups of cells from the main tumor mass. Actually, Coman (1947) has demonstrated that the force necessary to separate pairs of cells of squamous-cell carcinomas is much less than the force needed to pull apart pairs of normal epidermal cells.

The studies reported here on the physical-chemical properties of the urea and alkali soluble proteins of epidermis in various pathological states indicate possible trends only. The average mobility of the urea extractable protein of mouse epidermis undergoing carcinogenesis was highest for the carcinoma, and lowest for the croton oil and methylcholanthrene treated epidermis. Two samples of human basal-cell carcinomas were found to contain no measurable amount of urea extractable protein precipitable at pH 5·5, but the one which flocculated at pH 4·5 was isolated in the customary manner.

The s_{20} constant and intrinsic viscosity of the urea extractable protein were of the same order of magnitude for the protein isolated from croton oil and methylcholanthrene treated epidermis, papillomas and carcinomas. The s_{20} value of the alkali soluble protein was similar for these tissues, but there was a decrease in the average intrinsic viscosity for this protein in borate-6M urea buffer in the order croton oil and methylcholanthrene treated epidermis, papillomas and carcinomas. The schlieren peaks of the urea and alakli-soluble proteins were much sharper and the sedimentation constants were lower in the borate buffer-6M urea solutions than in the borate buffer alone. The latter difference is probably due to an increase in asymmetry of the protein molecules in the urea solutions.

SUMMARY

Certain urea and alkali-soluble proteins of mouse epidermis undergoing carcinogenesis and of human and beef snout epidermis were investigated.

The average mobility of the urea extractable protein of croton oil and methylcholanthrene treated epidermis was lower than that of the papillomas and carcinomas. The average mobility of the alkali soluble protein of these tissues was not appreciably different.

The average sedimentation constant of the urea and alkali soluble proteins of croton oil and methylcholanthrene treated epidermis, papillomas and carcinomas were not appreciably different. The average intrinsic viscosity of the alkali soluble protein was greater for the croton oil and methylcholanthrene treated epidermis than for the carcinomas. The average intrinsic viscosity of the urea extractable protein of these tissues showed no appreciable difference.

The sedimentation constant was less and the schlieren peaks were sharper for the epidermal proteins in 6M urea borate buffer solution than in borate buffer alone. The former difference is probably due to the protein molecules being more asymmetrical in the urea solution than in the borate buffer alone.

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THE INTRACELLULAR METABOLISM OF 3 : 4 BENZPYRENE: METABOLISM IN THE KIDNEYS AND SKIN OF RATS AND MICE

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THE metabolism of 3 : 4 benzpyrene within different structural components of liver cells has already been studied by Calcutt and Payne (1954*a*, 1954*b*, 1954*c* and 1954*d*). In relation to the problem of carcinogenesis the results recorded in these papers are open to the criticism that the liver in both rats and mice is effectively non-susceptible to the action of 3 : 4 benzpyrene. The studies have therefore been extended to cover kidney and skin.

Like the liver the kidney in rodents does not appear to be susceptible to hydro carbon carcinogenesis, though Ilfeld (1936) obtained a number of kidney tumours in mice after insertion of pellets of 1 : 2 : 5 : 6 dibenzanthracene. The reason for including this tissue in the present work is that Weigert and Mottram (1946) found unchanged benzpyrene and benzpyrene metabolites in the kidneys of mice which had received this hydrocarbon. These findings imply that metabolism of the hydrocarbon can normally occur in this organ.

Skin is an obvious choice of tissue since from the data summarised by Hartwell (1951) it is apparent that mouse skin readily responds to 3 : 4 benzpyrene by tumour induction, but that under similar experimental conditions rat skin is refractory to the hydrocarbon.

From the aspect of experiments involving the separation of cellular components kidney is a tissue which is easily handled and has been extensively used in work of this type. Skin on the other hand is very difficult to homogenise and even after homogenisation only two fractions are separable by centrifugation (Weist and Heidelberger, 1953). One is composed of unbroken cells and the particulate components of the disrupted cells whilst the other is a clear supernatant fraction. However, Fiala, Sproul and Fiala (1955) showed that adequate separations can be achieved if the skin of very young mice is used. This finding has been confirmed and also extended to cover skin from day-old rats. So the skin experiments reported later in this paper refer only to material derived from day-old animals.

EXPERIMENTAL

All tissues used in the experiments reported here were taken from RIII mice or Wistar rats. Benzpyrene used in kidney experiments was in the form of a colloid in distilled water at a concentration of 1 mg. per c.c. In the skin experiments a 1 per cent solution in acetone was used, this solvent being chosen on the basis of Pullinger's (1940) statement that cold acetone does not affect the microscopic structure of mouse skin.

Since the kidneys lie in the pathway of excretion via the urine the presence of a compound in this organ does not necessarily imply its formation at this site. The experiments concerned with metabolism in kidneys have therefore been carried out under *in vitro* conditions. Kidneys were removed from freshly killed rats or mice and homogenised in Tyrode or 0·88 M sucrose or 1 per cent citric acid solution. The homogenate was then divided by centrifugation into fractions comprising nuclei, mitochondria, microsomes and supernatant. After re-suspension and washing each fraction was re-suspended in a small volume of the dispersing medium together with some colloidal benzpyrene. The fractions were then placed in an incubator at 37° C. in total darkness. After suitable time intervals the fractions were removed and processed for benzpyrene metabolites as described by Calcutt and Payne (1954*b*). The metabolites sought were those described and labelled by Weigert and Mottram (1946) as BpX_1 and BpX_2. Identity of the metabolites was based on a correspondence of fluorescence colour, chromatographic behaviour and absorption spectra with those recorded by Weigert and Mottram (1946).

In the case of skin, the hydrocarbon in acetone solution was directly applied to the back and flanks of groups of mice or rats which were not more than 24 hours old. After varying time intervals the animals were killed, the skin stripped off with forceps and homogenised. After separation into nuclei, mitochondria, microsomes and supernatant by centrifugation the individual fractions were checked for the presence of benzpyrene metabolites as in the kidney experiments. In this series of experiments all homogenisations were carried out in 1 per cent citric acid as it was found that if 0·88 M sucrose or Tyrode were used almost the entire particulate fraction sedimented with the nuclei on subsequent centrifugation. With citric acid as the dispersing medium excellent separation of the individual cell fractions was obtained.

Parallel to the above experiments the distribution of unchanged benzpyrene within kidney and skin cells was also determined. In the case of kidney these organs were removed from animals which had previously received intravenous injections of colloidal benzpyrene. After homogenisation and centrifugal separation into cell fractions each fraction was extracted with acetone until no further fluorescent material was removed. The acetone was then boiled off from the pooled washes under reduced pressure and the residue extracted with cyclohexane. This was dried over anhydrous sodium sulphate and passed through a column of alumina. Any benzpyrene present was held as a blue-violet fluorescent zone on the column, and was readily eluted with ethyl alcohol. Final identification was by absorption spectroscopy.

Using skin the problem is complicated by the presence of residual dried hydrocarbon left after the painting. This can be removed by washing with organic solvents but this causes hardening of the tissue and leads to difficulty with homogenisation. The expedient was adopted of preparing cell fractions from hydrocarbon painted skin and then washing the individual fractions with acetone till no further benzpyrene could be removed. Any hydrocarbon remaining must be considered as firmly bound. Its release was effected by refluxing the fractions for two hours in 50 per cent hydrochloric acid in the presence of red phosphorus and potassium iodide, these being used in the proportions of 6 : 2·5 by weight and an obvious excess of the mixture was used with each fraction. Under these conditions benzpyrene was released from the fractions and collected in the

condenser from which it could be washed with cyclohexane in such a state of purity as to be ready for estimation by absorption spectroscopy.

RESULTS

Metabolism in kidney cell fractions

The findings in these experiments are shown in Table I. Under the *in vitro* conditions used benzpyrene was metabolised in all four cell fractions. The benzpyrene metabolite, BpX_2, was found in association with the nuclei, mitochondria and microsomes. In the supernatant fraction both BpX_1 and BpX_2 were present. These results are identical with those previously recorded by Calcutt and Payne (1954*b*, 1954*c* and 1954*d*) for rat and mouse liver.

TABLE I.—*Benzpyrene Metabolites in Kidney Fractions Incubated with Benzpyrene*

	Number of animals	Sex	Incubation period (hrs.)	Medium	Benpyrene metabolites in: Nuclei	Mitochondria	Microsomes	Supernatant	
Mice	12	M.	88	Tyrode	BpX_2	BpX_2	BpX_2	BpX_1	BpX_2
	12	F.	64	1% citric acid	BpX_2	BpX_2	BpX_2	BpX_1	BpX_2
Rats	3	M.	28	Tyrode	BpX_2	BpX_2	BpX_2	BpX_1	BpX_2
	3	F.	34	0·88 M sucrose	BpX_2	BpX_2	BpX_2	BpX_1	BpX_2

Although these results indicate that kidney cell fractions have the same potentialities for metabolising benzpyrene as the corresponding liver fractions they do not show that metabolism occurs within the kidney in the intact animal. If these findings are taken together with the previously mentioned evidence that benzpyrene is deposited in the kidney it appears likely that metabolism occurs in this organ.

Metabolism in skin

The skin from 34 day-old RIII mice which had been painted with 1 per cent benzpyrene in acetone $2\frac{3}{4}$ hours earlier was separated into nuclei, mitochondria, microsomes and supernatant. Each fraction was examined for benzpyrene metabolites. The derivative BpX_2 was found associated with all four fractions but no BpX_1 was detected.

In a comparable experiment with 14 day-old rats all four cell fractions contained BpX_2 but no BpX_1 was found.

This failure to find BpX_1 was interesting but in agreement with the earlier work of Weigert, Calcutt and Powell (1946, 1947) where BpX_2 only was detected in whole skin from benzpyrene treated mice. To attempt to obviate the possibility that BpX_1 is formed but only in very small amounts some further *in vitro* experiments were undertaken.

Fractions from the skins of 60 mice were incubated for 32 hours in the presence of benzpyrene and tetramethyluric acid. This last addition was based on Weil-Malherbe's (1946) evidence showing that tetramethyluric acid has a remarkable solvent power for 3 : 4 benzpyrene. It was hoped by this method to increase the penetration of the benzpyrene into the tissue fractions and thus increase meta-

bolism. From the subsequent extractions BpX_2 only was obtained; this being associated with all fractions. In corresponding experiments with the skin of 12 rats incubated for 72 hours similar results were obtained. A further experiment using skin from 13 rats (but without the addition of tetramethyluric acid) and incubated for 16 hours gave identical results.

Distribution of benzpyrene within kidney cells

These findings derived from the kidneys of rats or mice which had received previous intravenous injections of the colloidal hydrocarbon. The results are given in Table II.

TABLE II.—*Distribution of* 3 : 4 *Benzpyrene in Mouse and Rat Kidneys*

Bp = hydrocarbon present.
— = hydrocarbon absent.

	Number of animals	Sex	Time of killing (hrs.)	Dispersal medium	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	10	F.	1½	1% citric acid	Bp	Bp	Bp	Bp
	8	M.	4	0·88 M sucrose	Bp	Bp	Bp	Bp
	9	F.	16	Tyrode	Bp	Bp	—	—
Rats	2	M.	2	Tyrode	Bp	Bp	Bp	Bp
	2	F.	5½	0·88 M sucrose	Bp	Bp	Bp	Bp
	2	,,	18	1% citric acid	Bp	Bp	Bp	—

Distribution of benzpyrene within skin cells

It has already been shown by Fiala, Sproul and Fiala (1955) that benzpyrene penetrates to and is bound in nuclei, mitochondria, microsomes and supernatant of mouse skin cells. In the present experiments the hydrocarbon has been detected in all four fractions of rat skin cells 2½, 6 and 22 hours after painting with the hydrocarbon.

DISCUSSION

The results recorded in the foregoing paragraphs show a remarkable resemblance to those previously recorded by Calcutt and Payne (1954*b*, 1954*c*, 1954*d*) in respect of similar work involving liver cells.

As in the liver experiments the hydrocarbon was found to penetrate throughout the cell in both kidney and skin. The formation of benzpyrene X_2 takes place in all four cell fractions as in the liver, but although BpX_1 was found in the supernatant fraction from kidneys no trace of this derivative was obtained in skin. It can be said then that the intracellular behaviour of benzpyrene in kidney cells is identical with that in liver cells of both rats and mice. In skin cells, although the general pattern is similar, a distinction appears in that the metabolic derivative BpX_1 is no longer found.

Since BpX_1 is formed in both liver and kidney, where tumour induction by polycyclic hydrocarbons is a rare occurrence, but is not formed in skin which is susceptible to the carcinogenic action of benzpyrene it appears that this derivative can play no part in the carcinogenic process. Therefore, Boyland and Weigert's

(1947) proposal that BpX_1 or its immediate breakdown product, BpF_1, is the proximate carcinogenic agent is no longer tenable. BpX_1 would now appear to be a mere detoxication product.

Boyland (1950) has collected evidence indicative of a causal relationship between metabolism of carcinogens and their carcinogenic activity. If this is accepted it would now appear, in the light of the new evidence that the carcinogenecity of benzpyrene is associated with either the formation of the derivative BpX_2 or with the formation of some as yet unknown derivative. In relation to this second possibility Tarbell, Brooker, Seifert, Vanterpool, Claus and Conway (1956) have offered some evidence for a further, as yet unidentified, benzpyrene metabolite being formed in mouse skin.

Pullinger (1940) described nuclear abnormalities as occurring in mouse skin after application of benzpyrene, and it has now been shown that skin cell nuclei not only absorb the hydrocarbon but also metabolise it. Under these circumstances it is feasible that the nuclear derangements induced by benzpyrene are the result of activity within the nucleus itself.

An interesting point arising from the present work is the fact that no differences were detected in the behaviour of benzpyrene in rat or mouse skin cells. Yet the adult rat skin is refractory to hydrocarbon carcinogenesis whilst mouse skin is susceptible. The answer to this problem may lie in the fact that the present experiments have been concerned with skin from very young animals. Fiala, Sproul and Fiala (1955) stated that day-old mice painted with benzpyrene responded with tumour formation, but there appear to be no records of similar experiments with young rats.

The similarities of behaviour of benzpyrene in liver, kidney and skin cells as contrasted with the difference in biological response of skin as compared with the other tissues leaves further problems which require more experimental work.

SUMMARY

1. Benzpyrene has been found to be metabolised to the derivative BpX_2 in nuclei, mitochondria, microsomes and supernatant of kidney and skin cells from rats and mice.

2. The derivative BpX_1 was found to be formed in the supernatant fraction of kidney cells of both rats and mice but never to occur in skin.

3. Unchanged benzpyrene penetrates to nuclei, mitochondria, microsomes and supernatant of kidney and skin cells of both rats and mice.

4. It is concluded that the benzpyrene derivative, BpX_1, has no association with the carcinogenic activity of the hydrocarbon.

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THE NUCLEIC ACID OF PARTIALLY PURIFIED ROUS NO. 1 SARCOMA VIRUS

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ALTHOUGH tumour viruses have so far not been isolated in highly purified form, it seems likely that most of the material in the partially purified preparations obtained by repeated centrifugation and enzyme treatment (Carr and Harris, 1951; Bather, 1953) or centrifugation and protamine sulphate precipitation (Moloney, 1955) comes only from the cytoplasm of tumour cells. It is of interest therefore, to investigate the nucleic acid (N.A.) of such preparations using the newer methods of extraction and chromatography that have been applied with success to influenza virus (Ada and Perry, 1954). The present paper describes some preliminary observations on the chemical composition and nucleic acid content of Rous sarcoma virus concentrates and of cytoplasmic particle concentrates from normal fowl tissues.

MATERIAL AND METHODS

Chickens from the highly susceptible inbred line of Brown Leghorns maintained at this Centre were used throughout. These birds are constantly selected for susceptibility to Rous sarcoma cells and virus, and allow infectivity titrations to be made in young chicks to a constant end-point. No negatives appear in any of the groups preceding that inoculated with the highest active dilution. The method of Parker and Rivers (1936) can be used to estimate the second figure in the end-point dilution. Groups of 4 or 5 chicks were used for each serial tenfold dilution of virus.

Preparation of partially purified virus and cytoplasmic particulate concentrates

Most of the preparations were made by the standard method used in previous work (Bather, 1953) which was adapted from that originally devised by Carr and Harris (1951). The method employs fractional centrifugation and treatment with the enzymes trypsin and hyaluronidase. The relative centrifugal forces used throughout were 3500 g. for 20 minutes in a swinging cup rotor for clarification and 20,000 g. for 45 minutes in a Servall SS.1 angle head centrifuge for sedimentation of the virus and cytoplasmic particulates. On three occasions, protamine sulphate precipitation (Moloney, 1955) was used and gave final pellets which contained similar amounts of material to that obtained by the standard procedure (see " Experimental "). The concentrates were transferred to weighed test-tubes and stored in the deep-freeze until needed for further study. Infectivity titrations were always made on a sample of suspension immediately prior to the final sedimentation.

Isolation and estimation of lipid and nucleic acid

The virus or particulate concentrates were removed from the deep freeze and dried from the frozen state *in vacuo*. Lipids were extracted from the dried material with two washings of chloroform : ethanol mixture (2 : 1) followed by two washings with dry ether. The residue was dried *in vacuo* and weighed, and lipid content estimated by difference. The lipid extracts were pooled and in some cases the solvents were evaporated off and phosphorus determinations made on the residues. Five determinations made on Rous virus lipids gave an average of 2·2 per cent P with a range of 1·6–2·4 per cent. The average figure agrees closely with that found by Claude (1939) and indicates that approximately half of the lipid material is in the form of phospholipids. [Recent work of Moloney (1957) has pointed up the importance of oxidized phospholipids in the inactivation of Rous sarcoma virus.]

The lipid-free residues were extracted with 10 per cent sodium chloride at 100° C. for thirty minutes as described by Ada and Perry (1954). The residue was centrifuged after extraction and washed twice with 10 per cent sodium chloride. The extracts were pooled and diluted appropriately for reading in the Ultra Violet Spectrophotometer (Unicam SP.500). Readings were made over the wavelength range of 240–300 mμ and a standard curve was prepared using a purified sample (Smith and Markham, 1950) of commercial yeast nucleic acid. The purified yeast N.A. contained 8·16 per cent phosphorus. Blank readings were made on a 10 per cent sodium chloride extract of normal chicken plasma protein. The fit of the corrected U.V. absorption curves of virus and particulate N.A. were very close to that of the purified material (Fig. 1).

The salt extracted residues from three different virus preparations were further extracted with 1 N perchloric acid at 80° C. for thirty minutes. No evidence of residual N.A. was found in the perchloric acid extracts when examined in the U.V. Spectrophotometer (Fig. 1).

Estimates of the amounts of N.A. in the NaCl extracts were made by applying the factor 9·7 calculated from the known proportions of bases (see " Experimental ") to convert their phosphorus content to N.A. content.

Determination of the molecular proportions of nitrogenous bases in the nucleic acids

The N.A. extracted as described was precipitated from the 10 per cent NaCl solution by the addition of two volumes of 96 per cent ethyl alcohol and a few drops of 0·1 N HCl to reduce the pH to approximately 2. Precipitation was allowed to take place at 0–4° C. for at least 24 hours. The precipitates were washed in 66 per cent, then 96 per cent ethanol and, finally, dry ether, and dried. 1·0 N HCl was added so that the final concentration of N.A. was about 10 mg./ml. (usually 40–50 μl. were required) and the tubes sealed. Hydrolysis was carried out at 100° C. for one hour. The hydrolysates were spotted on paper strips washed according to the method of Hanes, Hird and Isherwood (1952). The strips were usually 1·0–1·5 cm. wide. The hydrolyzed bases were separated by ascending chromatography in the solvent mixture isopropanol—HCl—water (65–18–17) (Wyatt, 1951).

The bases were located by U.V. light using a Chromatolite Fluorescence Lamp (Scottish Instruments) and cut out, together with corresponding pieces from a blank strip and eluted in 5 ml. 0·1 N HCl for 24 hours. A sample of hydro-

lyzed yeast N.A. was always run with each series of virus or particulate N.A.'s. The concentration of each purine or pyrimidine nucleotide was estimated by its absorption in the U.V. Spectrophotometer. Absorption at the maxima were

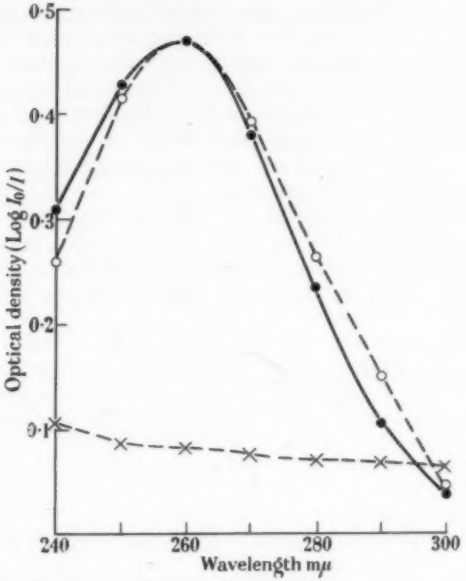

FIG. 1.—Ultra violet absorption curves of purified yeast R.N.A., partially purified Rous virus R.N.A. and perchloric acid extract of salt extracted Rous virus preparation.

●——● Partially purified Rous virus R.N.A.
○- - - -○ Purified yeast R.N.A.
×- - - -× Perchloride acid extract of salt extracted Rous virus preparations.

determined for 10^{-3} M solutions or each of the purine bases and pyrimidine nucleotides using pure preparations (Light & Co.).

The values obtained were :

Guanine	E_{250}	= 11·0
Adenine	E_{260}	= 13·0
Cytidylic acid	E_{280}	= 12·5
Uridylic acid	E_{260}	= 10·0

Other procedures will be discussed in the experimental section concerned.

EXPERIMENTAL

Examination of the partially purified virus preparations in the electron microscope and the analytical ultra centrifuge

Samples of the partially purified virus preparations were frequently taken after the final sedimentation. These were resuspended in water or phosphate-

citrate buffer at pH 6·7 and sprayed on to grids for examination in a Phillips (25Å Model) Electron Microscope or a Spinco electrically driven analytical ultra-centrifuge.

The electron micrographs usually showed particles of a fairly uniform size distribution. In some preparations large, diffuse spots were present which tended to fade at high voltage and may have been free lipoid material. No attempt at accurate measurement of the particles was made because of generally poor contrast and uncertainty as to the true nature of all the particles present. However, electron microscope examination of many of the concentrated preparations served to confirm a reasonable uniformity of particle size within the range expected for Rous sarcoma virus (50–100 mμ) and it was not considered feasible at the present stage to expect more detailed information.

Rates of sedimentation of the partially purified virus preparations were determined using the Spinco ultra-centrifuge incorporating a modified Schlieren optical system. Experiments were carried out on highly active preparations of several concentrations between 0·05 and 0·3 per cent in 0·1 M phosphate : citrate buffer (pH 6·7). Speeds of 12,500 r.p.m. were used.

Within experimental error the sedimentation constant was independent of concentration and had the value of 470×10^{-13} C.G.S. units. Using the newer value of Kahler, Bryan, Lloyd and Moloney (1954*a*) for the density of Rous sarcoma virus this leads to an average diameter for the particles of approximately 80 mμ.

Presence of normal cell components in partially purified Rous sarcoma virus preparations

The normal cell contaminants most likely to be present after careful selection of viable tumour tissues are from blood, muscle fibres and, possibly, connective tissue cells. The author has observed that muscle cells yield only about 1/10 as much concentrated cytoplasmic material as tumour. Red blood cells contain little of this type of particle in their cytoplasm. White cells and lymphocytes may be expected to yield a considerable amount of cytoplasmic concentrate as is evident from the results for spleen ("Experimental" Section 4) but the numbers of these cells present must be small. There remains the possibility that, since Rous sarcoma is of connective tissue origin, there may remain in the tumour cells cytoplasmic material derived from normal connective tissue cells, which may contribute particles to the virus concentrates. Attempts were made to isolate a cytoplasmic particle concentrate from cock's comb, a tissue that is almost entirely composed of connective tissue cells. Microscopically, comb tissue presents a picture reminiscent of spindle-cell sarcoma with whorling bundles of connective tissue cells and, like the avian virus-induced tumours, yields considerable amounts of mucoid material which is degraded by hyaluronidase. As much as 48 g. of pooled comb tissue were homogenized and extracted in the same way as tumour tissue. The amount of concentrated material in the final pellet was minute and difficult to disperse. It was found to consist almost entirely of collagen fibres whose characteristic banded structure was clearly seen in the electron microscope.

It is clear, then, that any contribution by normal particles to the virus concentrates must be very small and even a figure of 1 per cent is probably too high.

Identification of the purine-bound sugar in the nucleic acid of partially purified Rous virus preparations

Three Rous virus concentrate preparations were analysed for the presence of deoxyribose by the method of Webb and Levy (1955) using *p*-nitrophenylhydrazine. No deoxyribose was detected despite the reported high sensitivity of this reagent.

The purine-bound sugar was released from partially purified Rous virus nucleic acid (prepared as described in "Methods") by digestion of approximately 500 μg. N.A. in 0·25 ml. normal H_2SO_4 in a sealed tube at 100° C. for one hour. The hydrolysate was neutralized with saturated $Ba(OH)_2$ and the supernatant evaporated to dryness. The resulting residue was extracted with hot 96 per cent ethanol and the extract evaporated to a small enough quantity for convenient spotting for paper chromatography. Two solvent systems were used, namely phenol and butanol-acetic acid. The isolated sugar was chromatographed with pure ribose, xylose and arabinose. In three experiments the purine-bound sugar from Rous virus concentrates corresponded in R_F value with pure ribose. No deoxyribose or other purine sugar was evident.

Lipid and ribonucleic acid content of partially purified Rous virus preparations and normal cytoplasmic particulate concentrates

Lipids and nucleic acids were isolated and estimated as described in "Methods". The results are listed in Table I. The first set of values for Rous sarcoma are for concentrates prepared by the standard fractional centrifugation-enzyme digestion procedure described in the "Methods". The second set are for concentrates prepared as described by Moloney (1955) using protamine sulphate precipitation.

TABLE I.—*Lipid and Ribonucleic Acid Content of Partially Purified Rous Sarcoma Virus Preparations and Cytoplasmic Particulate Concentrates from Spleen, Liver and Brain*

Tissue of origin	Observations	Virus and particulate yields mg./g. wet tissue	Per cent lipid ± S.D.	Per cent R.N.A. ± S.D.	Range of infectivity (M.I.D./g. tumour)
Rous sarcoma (1)	38	0·51	42·5 ±9·0	1·42 ±0·37	$10^{1.2}$–$10^{3.5}$
Rous sarcoma (2)	3	0·54	43·6* (37·2–50·0)	0·78* (0·92–0·64)	$10^{2.5}$–$10^{4.5}$
Spleen	4	7·04	36·5 ±4·0	1·02 ±0·10	—
Liver	5	4·60	37·6 ±4·7	0·61 ±0·14	—
Brain	2	5·96	31·6 (32·7–30·4)	0·44 (0·42–0·46)	—

* Two determinations only.

Both sets of results are comparable, although the nucleic acid content is low in the protamine sulphate preparations. However, there is a wide variation in the R.N.A. content of tumour extracts prepared by either method (0·64–2·12 per cent) and this may be a reflection of the variation in infectivity of the different preparations. The range of infectivities available is not sufficient, as yet, to show whether

or not a correlation exists with R.N.A. content of the type found for influenza virus (Ada and Perry, 1956). Liver and brain concentrates appear to have a lower average content of R.N.A. compared with Rous virus concentrates, brain showing the lowest. This could be due to the lesser activity of these tissues metabolically (especially in the case of brain) assuming that R.N.A. is intimately concerned in growth and cell division.

Molecular proportions of nitrogenous bases in ribonucleic acid from various sources

Ribonucleic acid isolated from partially purified virus preparations and normal cell particulate concentrates was hydrolyzed and chromatographed as described in the "Methods". The molecular proportions, referred to Adenine as 1·00, were calculated from the U.V. absorption data of the eluted paper strips (Table II).

TABLE II.—*Molecular Proportions of Purine and Pyrimidine Bases obtained by Chromatographic Separation on Filter Paper of R.N.A. from Yeast, Partially Purified Rous sarcoma Virus Preparations and Cytoplasmic Concentrates of Fowl Liver and Spleen*

Source of R.N.A.	Number of obs.	Adenine	Guanine ± S.D.	Cytosine ± S.D.	Uracil ± S.D.	$\frac{A+U}{G+C}$ ± S.D.	$\frac{Pu.}{Py.}$ ± S.D.	$\frac{A+C}{G+U}$ ± S.D.
Yeast .	7	1·00	1·15	0·82	0·88	0·96	1·27	0·89
			±0·04	±0·01	±0·02	±0·02	±0·03	±0·02
Rous sarcoma	6	1·00	1·80	1·55	0·81	0·54	1·20	0·98
			±0·08	±0·03	±0·07	±0·03	±0·04	±0·03
Spleen .	4	1·00	1·98	1·54	0·81	0·52	1·27	0·92
			±0·11	±0·13	±0·08	±0·01	±0·05	±0·06
Liver .	2	1·00	1·81	1·58	0·71	0·50	1·22	1·02
			(1·78–1·84)	(1·62–1·54)	(0·67–0·75)	(0·49–0·52)	(1·22–1·24)	(1·07–0·98)

R.N.A. from Rous sarcoma concentrated extracts in common with that from spleen and liver, contains a large proportion of both guanine and cytosine. Such a preponderance of these two bases has been found in most ribonucleic acids studied with the exception of yeast and influenza virus R.N.A. (Elson and Chargaff, 1954; Ada and Perry, 1954, 1956). No significant differences have appeared in the ratios of $\frac{\text{adenine} + \text{uracil}}{\text{guanine} + \text{cytosine}}$ between Rous virus concentrate and spleen and liver R.N.A. when subjected to the *t*-test at the 1 per cent level. The final column $\left(\frac{\text{adenine} + \text{cytosine}}{\text{guanine} + \text{uracil}}\right)$ indicates that in all cases the number of 6-keto groups is nearly equal to the number of 6-amino groups and is of interest in relation to the possible configuration of the R.N.A. molecule (Elson and Chargaff, 1954).

DISCUSSION

Previous estimates of the nucleic acid content of partially purified Rous virus concentrates were made by Claude (1939), who gave a value of 5–8 per cent of the total virus material as N.A., and by Shemin and Sproule (1942) who estimated the N.A. as approximately 1·5 per cent. The former based his values on U.V. absorption methods while the latter used purine nitrogen values. The experiments described here show that Rous virus concentrate R.N.A. can be

extracted completely and in a relatively pure form with 10 per cent NaCl and that the amounts so obtained vary considerably between a minimum of 0·64 per cent and a maximum of 2·12 per cent of the total virus concentrate. Claude did not use trypsin digestion to further purify his material and it may be that the high value found was because of this.

Recent reports from Kahler *et al.* (1954*a*, *b*) have placed the density of Rous virus at approximately 1·15 or lower and the diameter at 90 mμ. The data presented here indicate that the great majority of the particles in the partially purified virus preparations, with which almost all the infectivity is associated, are of fairly uniform size and density, as far as can be ascertained with the electron microscope and the analytical ultracentrifuge. Their diameter in these experiments was calculated as approximately 80 mμ. The molecular weight of the Rous virus particle has been estimated to be approximately 140×10^6 (Claude, 1937). The values for R.N.A. content given in this paper show that Rous sarcoma virus fits in with the relationship pointed out by Frisch-Niggemeyer (1956) and Cheng (1957), namely that most R.N.A. containing viruses and cytoplasmic particles appear to contain the same absolute amount of R.N.A. (approximately 2×10^6 g. per mole of virus or particle).

No differences in the molecular proportions of purine and pyrimidine bases occurred between partially purified preparations of Rous virus and the cytoplasmic particulate concentrates of the normal tissues studied. The question of purity is, of course, ever present in tumour virus work and it may be argued that most of the nucleic acid came from non-tumour sources. It can be said, however, that normal cell components must be present in very small amounts in virus concentrates prepared in this way. The fact that no D.N.A. can be detected in the preparations makes improbable contamination by nuclear material. Non-virus protein contaminants are removed to a large extent by trypsin treatment. We are left, then, with a suspension of cytoplasmic particles derived almost entirely from tumour cells, a counterpart of which (cock's comb connective tissue) yields negligible quantities of such particles. The fact that the amounts of material in the high speed centrifuge pellets are different when isolated from different tissues probably reflects the variations in the fine structure of the cytoplasm. Palade (1955) and Palade an Siekevitz (1956) have studied many types of cell in the electron microscope and attempted to relate the fine structure to cytological, histochemical and cytochemical information. It is not known how much of the pellet material obtained in these experiments can be attributed to the very small (100–150Å) particulates described by Palade. If, as has been suggested, these particulates correspond to those isolated by Petermann and co-workers by differential centrifugation (1952, 1953 and 1954) then most of them would be discarded during the isolation of the partially purified virus and cytoplasmic particulates by the method employed here. In any case, as Petermann and co-workers have shown that in mouse leukaemia all their spleen cytoplasmic particulate fractions increased over the normal picture it is probable that particles isolated along with the virus are closely related to virus in the metabolic processes of the cell. The situation is further complicated by the fact that only a very few particles structurally resembling virus are seen in sectioned pellets of centrifuged extracts of Rous sarcoma (Bernhard, Oberling and Vigier, 1956). No guarantee, therefore, that all the nucleic acid isolated from the partially purified virus preparations is from virus can be given. It can only be said that it is intimately associated with the cytoplasmic

particulates of Rous cells and with that fraction of the particulates which contains the infective principle.

It would be interesting to see if other virus-induced tumours yield concentrates containing different R.N.A. from that found in Rous, and several tumours which are now available at this Centre are being studied.

The lack of any marked difference between normal and virus nucleic acid may not be surprising when it is remembered that tumour viruses live in a peculiarly close symbiosis with the parent cell, usually remaining within the cell throughout the life of the tumour. Any differences there are in nucleic acid composition, therefore, may be so subtle as to be impossible to detect by methods such as were employed here.

SUMMARY

The nucleic acid associated with partially purified preparations of Rous sarcoma virus can be quantitatively extracted and estimated using 10 per cent sodium chloride solution and ultra-violet spectrophotometry. The preparations used consist of particles of reasonably uniform size exhibiting a sharp peak in the analytical ultracentrifuge and having a sedimentation constant of 470 C.G.S. units and a calculated diameter of approximately 80 mμ. They are almost entirely free of normal tissue and nuclear contaminants.

The purine-bound sugar contained in the nucleic acid obtained from the Rous sarcoma virus preparations has been identified by paper chromatography as ribose and no evidence of deoxyribose was found by chromatography or the *p*-nitrophenylhydrazine reaction.

A series of 38 Rous sarcoma virus preparations was found to contain 1·42 $\pm$ 0·37 per cent R.N.A. with a range of values extending from 0·64–2·12 per cent. The average R.N.A. content was higher than that of cytoplasmic particulate concentrates from spleen (1·02 $\pm$ 0·10 per cent), liver (0·61 $\pm$ 0·14 per cent) and brain (0·42–0·46 per cent).

No significant differences were found in the molecular proportions of purine and pyrimidine bases from Rous sarcoma virus preparations or cytoplasmic particulate concentrates from normal tissues.

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THE METABOLISM OF NEOPLASTIC TISSUES: THE INTERRELATIONSHIP BETWEEN OXIDATIVE AND GLYCOLYTIC MECHANISMS IN ASCITES TUMOUR CELLS

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ONE of the most interesting aspects of the metabolism of ascites tumour cells is their moderately high endogenous respiration which is depressed in the presence of glucose ("Crabtree effect") (Kun, Talalay and Williams Ashman, 1951). This phenomenon is the reverse of the classical Pasteur effect. Racker (1956) and Chance and Hess (1956) have argued that the Crabtree effect might be due to a shuttling of adenine nucleotides between the cytoplasm and the mitochondria, while Brin and McKee (1956) have shown that the concentration of inorganic phosphate might condition the inhibition of respiration by glycolysis. The sharing of these compounds between the two processes of carbohydrate breakdown has also been advocated to explain the Pasteur effect (Johnson, 1941; Lynen, 1941, 1956), but convincing evidence is still lacking (Aisenberg, Reinafarje and Potter, 1957).

The depression of the oxygen consumption brought about by glucose addition to the ascites cells is accompanied by a shift in the respiratory quotient from 0·85 (indicative of lipid oxidation) to 1·0 (Seelich, Weigert and Letnansky, 1956; Slechta, Jakubovic and Sorm, 1956). Slechta *et al.* (1956), and especially Bloch-Frankenthal and Weinhouse (1957), have furnished further evidence that the endogenous respiration of ascites cells might be due to fatty acids.

In the present investigation the interrelationship between the oxidative and glycolytic mechanisms in ascites tumour cells has been studied using C^{14}-labelled acetate, bicarbonate and amino acids *in vitro*.

MATERIALS AND METHODS

The rhabdomyosarcoma and mammary carcinoma ascites tumour which had been put at our disposal by Dr. G. Klein (Stockholm) were grown in $F_1(B \times C_3H)$ and $F_1(C_3H \times A)$ mice, respectively. After 8 days cells and ascites fluid were harvested and diluted with the same volume of physiological saline. After some preliminary centrifugations at low speed, the supernatant was centrifuged at $1200 \times g$ for 10 minutes at 0° C. Seven hundred to 750 mg. wet weight of the packed cells were transferred to 20 ml. incubation flasks containing 2·5 ml. Krebs-Ringer phosphate buffer at pH 7·4 and 1·25 mg. sodium acetate-1-C^{14}. Glucose was added as indicated. The radioacetate was synthesized from $BaC^{14}O_3$ and diluted for the present experiments to 5×10^5 counts per minute (c./m.), assayed as an "infinitely" thick layer of $BaC^{14}O_3$ on 1·1 cm.^{2} area under the Geiger-Müller end-window counter. Incubation was

carried out for 3 hours at 37° C. in an atmosphere of 100 per cent O_2. In a number of experiments the rhabdomyosarcoma was grown in the solid form by subcutaneous grafting of the ascites cells. Five hundred mg. wet weight of slices of this and other solid tumours were incubated under the conditions as described above. Isolation of the carbon dioxide, trichloroacetic acid-insoluble proteins and the long-chain fatty acid has been described (Emmelot and Bosch, 1955*a*). The digitonin precipitable steroid fraction (mainly cholesterol) was also isolated from the ascites cells but contained only a very small amount of C^{14}.

The proteins, fatty acids and respiratory $BaCO_3$ were plated on 1·1 cm.^{2} area and assayed at " infinite thickness " ; if necessary, corrections for self absorption were made. The incorporation of radioacetate into the fatty acids was calculated using the appropriate conversion factors.

In the experiments carried out with $NaHC^{14}O_3$ (synthesized from $BaC^{14}O_3$), DL-glutamate-1-C^{14} and DL-leucine-1-C^{14}, 350 mg. of packed mammary carcinoma ascites cells were incubated in 1·25 ml. Krebs-Ringer phosphate buffer for 2 hours. The ascites cells used in these experiments had been grown in $F_1(B \times C_3H)$ mice.

Oxygen consumption was measured by the standard Warburg technique.

$BaC^{14}O_3$, DL-glutamic-acid-1-C^{14} and DL-leucine-1-C^{14} were obtained from the Radiochemical Centre, Amersham.

RESULTS AND DISCUSSION

The ascites cells of the rhabdomyosarcoma and the mammary carcinoma used in the present investigation showed a linear oxygen uptake during the three-hour period which was chosen for the large scale experiments with radio-acetate. The oxygen consumption, measured with 100 mg. wet weight of cells, in the presence of acetate (2·2 μmole) and glucose (11 μmole) was 35–50 per cent lower than that in the presence of acetate alone.

The experiments in which 700–750 mg. freshly harvested rhabdomyosarcoma cells were incubated in the presence of sodium acetate-1-C^{14} (15·6 μmole) with and without unlabelled glucose (75 μmole) added, are illustrated in Table I. It is seen that the amount of carbon dioxide produced in the presence of glucose was always lower than that produced in the absence of glucose. In contrast, the specific activity of the barium carbonate was always higher in the presence of glucose. The total recovery of ^{14}C in the carbon dioxide from the glucose supplemented cells was enhanced in two experiments and depressed in the remaining three. This fall in the C^{14} recovery was, however, insignificant, amounting at its most to about 10 per cent of the total radioactivity recovered in the carbon dioxide in the absence of glucose. The data on the carbon dioxide demonstrate that the oxidation of the 2-C fragments *per se* is hardly affected by the glucose supplementation. Therefore another oxidative process must have been inhibited in the presence of glucose. To account for the marked effect on the oxygen consumption the latter could only have been the oxidative degradation of the fatty acids to their 2-C fragments. As a result a much smaller amount of unlabelled 2-C fragments from the fatty acids is supplied to the citric acid cycle. The unlabelled 2-C fragments which are still oxidized by the glucose-supplemented cells are derived to a marked extent from glucose (Bloch-Frankenthal and Weinhouse, 1957), but in the present case the latter oxidations are quanti-

tatively less than those of the 2-C fragments derived from the fatty acids in the absence of glucose. This is illustrated by the fall in the amount and the rise in the specific activity of the carbon dioxide recovered in the presence of glucose.

TABLE I.—*Effect of Unlabelled Glucose on the Incorporation of C^{14} from Acetate-1-C^{14} into the Carbon Dioxide, Long-chain Fatty Acids and Proteins of Rhabdomyosarcoma Ascites Cells*

700–750 mg. fresh weight of tumour cells incubated with 1·25 mg. sodium-acetate-1-C^{14} (A*) in 2·5 ml. Krebs-Ringer phosphate buffer at pH 7·4 for 3 hr. at 37° C. 75 μmole glucose (G) added as indicated.

Exp. No.	Addition	Carbon dioxide: $BaCO_3$ mg.	Carbon dioxide: $BaCO_3$ c./m.	Carbon dioxide: m-mole acetate incorporated $\times 10^3$	Fatty acids m-mole acetate incorporated $\times 10^5$	Proteins c./m.
1	A*	18·4	12,140	1·13	1·13	280
	A*+G	12·0	16,484	1·03	2·37	68
2	A*	14·8	11,103	0·82	0·14	279
	A*+G	10·0	14,700	0·76	1·80	70
3	A*	11·2	9,957	0·52	0·88	225
	A*+G	7·3	16,470	0·61	2·27	51
4	A*	13·6	11,670	0·80	1·13	285
	A*+G	9·3	15,750	0·75	2·68	86
5	A*	12·4	12,024	0·77	0·98	328
	A*+G	11·3	16,175	0·94	2·93	102

It may be expected (compare Table V)* that the tracer contained in the proteins after incubation with radioacetate is to a considerable extent localized in the amino acids belonging to the glutamate and aspartate families. The carbon skeletons of these amino acids are directly derived from the citric acid cycle intermediates α-ketoglutarate and oxaloacetate. Consequently, the extent of oxidation of radioacetate to carbon dioxide does not give a true measure of the amount of acetate funnelled through the cycle because of this loss of intermediates from the cycle. However, simple calculation (total activity contained in the proteins as c./m. $BaCO_3$) showed that the radioactivity thus withdrawn from oxidation, amounted to less than 10 per cent of the total amount of C^{14} recovered in the carbon dioxide in the absence of glucose. It follows then from these and the above results that the passage of radioacetate through the citric acid cycle is not or only slightly less (0–20 per cent) in the presence than in the absence of glucose.

Although the oxygen consumption of the mammary carcinoma ascites cells was markedly diminished in the presence of glucose, as mentioned above, the amount of carbon dioxide recovered as mg. $BaCO_3$ was not affected in most of the experiments (Table II). Since the specific activity of the carbon dioxide produced from acetate-1-C^{14} did not rise, the oxidation of acetate was practically

* If it is assumed that the proteins contain 15 per cent glutamate and 10 per cent aspartate, it can be calculated that approximately 70 per cent of the radioactivity of the proteins resides in these amino acids.

unaltered in the presence of glucose. The difference between the two tumours in respect to the CO_2 production may have been due to the fact that more unlabelled glucose was oxidized relative to acetate-1-C^{14} by the mammary carcinoma than by the rhabdomyosarcoma cells; the terminal oxidation of the 2-C fragments which would have been derived from the fatty acids being compensated by the oxidation of the 2-C fragments from glucose in the case of the mammary carcinoma. This assumption receives support from the fact that the total amount of acetate-1-C^{14} oxidized by the latter was on the average only one-third of that oxidized by the former tumour cells.

TABLE II.—*Effect of Unlabelled Glucose on the Incorporation of C^{14} from Acetate-1-C^{14} into the Carbon Dioxide, Long-chain Fatty Acids and Proteins of Mammary Carcinoma Ascites Cells*

Conditions: compare Table I.

Exp. No.	Addition	Carbon dioxide: $BaCO_3$ mg.	Carbon dioxide: $BaCO_3$ c./m.	Carbon dioxide: m-mole acetate incorporated $\times 10^3$	Fatty acids: m-mole acetate incorporated $\times 10^3$	Proteins c./m.
1	A*	11·9	2890	0·18	0·11	125
	A*+G	11·4	3011	0·18	1·03	61
2	A*	14·2	4112	0·30	0·26	136
	A*+G	13·1	3407	0·23	1·18	57
3	A*	13·1	3665	0·25	0·27	137
	A*+G	10·0	3930	0·20	1·01	72
4	A*	11·6	3425	0·20	0·14	127
	A*+G	10·9	3452	0·19	1·55	63

As might be expected, the incorporation of acetate-1-C^{14} into the long-chain fatty acids of the ascites cells was relatively poor in the absence, but markedly stimulated in the presence of glucose (Tables I and II). This stimulation is not characteristic of the ascites cells since it has been found also with many solid tumours (Emmelot and Bosch, 1955*b*; van Vals and Emmelot, 1957).

Incubation of the ascites cells with glucose and acetate-1-C^{14} resulted in a very marked drop in the C^{14}-content of the proteins as compared with the concentration of C^{14} found in the proteins after incubation with the radioacetate alone (Tables I and II). The average drop in the C^{14}-content of the proteins of the glucose supplemented mammary carcinoma cells was somewhat less than that found with the rhabdomyosarcoma ascites cells, *i.e.* 50 and 72 per cent respectively. This phenomenon has never been observed with any tumour of the solid type (Emmelot and Bosch, 1955*b*; van Vals and Emmelot, 1957). To see at which concentration of glucose the impairment in the incorporation of C^{14} into the proteins took place, two experiments were carried out with acetate-1-C^{14} and varying amounts of unlabelled glucose (19, 38, 75 and 113 μmole) using the rhabdomyosarcoma cells. The results are illustrated in Fig. 1. At the lowest concentration of glucose (one-fourth of that used in the experiments of Table I) the C^{14}-content of the proteins was already markedly diminished, at the next higher concentration this effect was more marked and then remained constant.

By contrast, the stimulation of fatty acid synthesis was, in both experiments, the most pronounced at the lowest glucose concentrations tested. The specific activities of the carbon dioxide were higher at all glucose concentrations and the total amount of acetate-1-C^{14} oxidized was only in one experiment with the 75 μmole concentration of glucose somewhat smaller than the values found in the absence of glucose.

It is known that pyruvate does not give rise to the Crabtree effect in ascites tumour cells (Racker, 1956). Unlabelled pyruvate (75 μmole), added to mammary ascites cells (750 mg.), and incubated with radioacetate for 2·5 hours, caused a

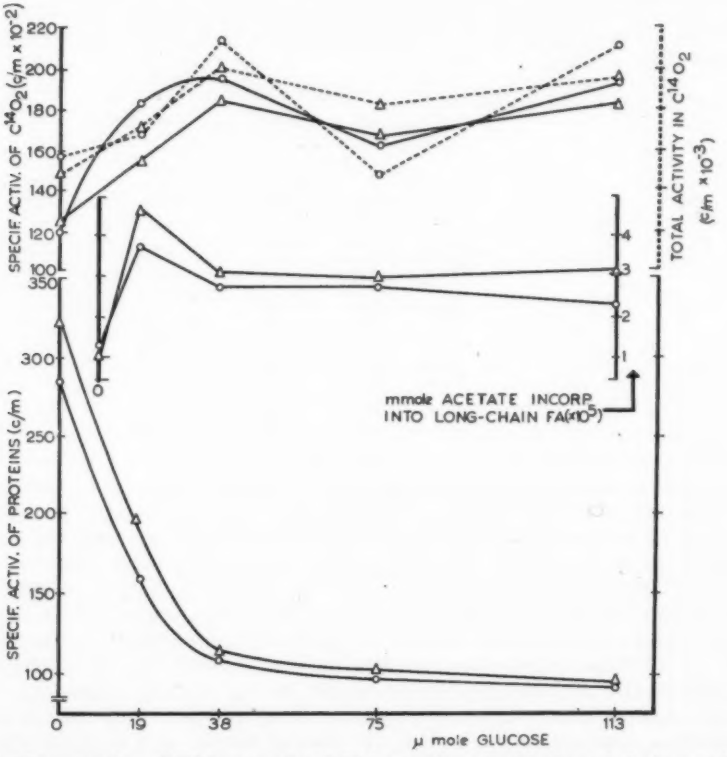

FIG. 1.—Incorporation of C^{14} from acetate-1-C^{14} into carbon dioxide, long-chain fatty acids (FA) and proteins by ascites cells of a rhabdomyosarcoma in the presence of various concentrations unlabelled glucose.

The results of two experiments are illustrated: △—△ with ascites cells containing 10·8 mg. N (Kjeldahl) and ○—○ *ibidem* 11·0 mg. N. Specific activity of $C^{14}O_2$ (solid lines) and proteins measured as counts per minute of an "infinitely" thick layer of $BaCO_3$, or protein on 1·1 cm.² area.

For convenience of representing the graphs, the total activity in $C^{14}O_2$ (dotted lines) is expressed as specific activity × mg. $BaCO_3$ recovered. Calculation showed that 0·77 (glucose absent), 0·88 (19 μmole glucose present), 1·05 (38 μmole glucose present), 0·94 (75 μmole glucose present) and 0·99 (113 μmole glucose present) × 10^{-3} m-mole acetate were incorporated into the carbon dioxide samples of the first experiment, and respectively 0·80, 0·87, 1·10, 0·75 and 1·08 × 10^{-3} m-mole acetate in the second experiment.

five-fold drop in the C^{14}-content of the proteins (from 110 to 25 c./m.). A corresponding fall was noted in the specific activity of the respiratory carbon dioxide: 12·4 mg. $BaCO_3$ showing 3047 c./m. at "infinite thickness" was recovered in the absence, and 17·8 mg. $BaCO_3$ showing 600 c./m. in the presence of pyruvate. The drop in the C^{14}-content of the proteins following pyruvate supplementation was therefore due to a dilution of the citric acid cycle intermediates which yielded the carbon skeletons of the amino acids incorporated into the proteins.*

The pattern of acetate utilization by the rhabdomyosarcoma cells in the presence of glucose was not likely to be due to the particular origin of this tumour, because the C^{14} incorporation into both the proteins and carbon dioxide of slices from normal thigh musle was always found to be stimulated in the presence of unlabelled glucose. In contrast to the depressive effect of glucose on protein labelling by acetate-1-C^{14} in the ascites cells of the rhabdomyosarcoma, no such effect was observed with the solid form of this tumour (Table III). Similarly, only a very small drop (if any) in the C^{14}-content of the proteins of spontaneous or transplanted mammary carcinomas from C_3H mice was noted in the presence of unlabelled glucose.

TABLE III.—*Effect of Unlabelled Glucose on the Incorporation of C^{14} from Acetate-1-C^{14} into the Carbon Dioxide, Long-chain Fatty Acids and Proteins of Slices from the Solid Rhabdomyosarcoma*

Addition	Carbon dioxide m-mole acetate incorporated $\times 10^3$	Fatty acids m-mole acetate incorporated $\times 10^5$	Proteins c./m.
A*	0·29	0·67	150
A*+G	0·23	4·02	150

The depressive effect of glucose on the incorporation of tracer from acetate-1-C^{14} into the proteins was thus characteristic of the ascites form of the present tumours and might in some way be connected with the concomitant shift in the utilization of the pathways of intermediary metabolism.

As follows from the CO_2 data collected in Tables I and II, and Fig. 1, the drop in the C^{14}-content of the proteins could not have been due to a dilution of the tracer in the citric acid cycle intermediates by unlabelled 2-C fragments derived from glucose.

Two other possibilities were, therefore, tested: the impairment by glucose of (i) the formation of certain C^{14}-amino acids (probably those derived from the citric acid cycle intermediates), and (ii) the incorporation of these amino acids into the proteins.

Since the incorporation of DL-glutamate-1-C^{14} and DL-leucine-1-C^{14} into the proteins of the mammary carcinoma ascites cells, was essentially the same whether glucose was present or not† (Table IV), the second of these alternatives could be discarded.

* The radioactivity of the long-chain fatty acids was five times higher (557 c./m.) after incubation in the presence of pyruvate than that found in the absence of pyruvate (117 c./m.).

† Incidentally, these experiments show that in these ascites cells the energy for the incorporation process can be derived as well from fatty acid as from glucose catabolic pathways. The incorporation of the amino acids into the proteins of slices from the sarcomatoid ovarian tumour T 26567$_s$, on the other hand, was found to be stimulated in the presence of glucose—more than 2-fold in the case of glutamate and 1·5-fold in the case of leucine.

TABLE IV.—*Effect of Glucose on Protein Labelling by and Oxidation of Acetate-1-C^{14}, DL-leucine-1-C^{14}, and DL-glutamate-1-C^{14} in Mammary Carcinoma Ascites Cells*

300 mg. packed ascites cells incubated for 2 hours in 1·25 ml. Krebs-Ringer phosphate buffer. G = glucose (37 μmole); A = sodium acetate (7·6 μmole); A* = sodium acetate-1-C^{14} (2 μC/7·6 μmole); Gl* = DL-glutamic acid-1-C^{14} (0·33 μC/0·25 μmole); L* = DL-leucine-1-C^{14} (0·33 μC/0·28 μmole).

Exp. No.	Additions	Protein c./m.	Carbon dioxide	
			mg. $BaCO_3$	c./m.
1	A*	131	6·0	3709
	A*, G	47	5·1	4138
1	L*, A	805	7·2	2268
	L*, A, G	797	5·7	550
2	L*, A	879	7·2	2082
	L*, A, G	875	6·5	344
1	Gl*, A	26	6·7	1457
	Gl*, A, G	26	5·7	3238
3	Gl*, A	31	6·6	1589
	Gl*, A, G	32	6·1	4652

It remained then to be demonstrated that the shift from fatty acid to glucose catabolism in the ascites cells was really accompanied by an impairment in the formation of certain amino acids. To this end the nature of these amino acids had to be ascertained. This problem was approached in two ways. First, it was demonstrated that the glucose-induced fall in the protein labelling by acetate-1-C^{14} was accompanied by a fall of comparable magnitude in the radioactivity of glutamate and aspartate isolated from the protein hydrolysates (Table V). Secondly, it was shown that the fixation of $NaHC^{14}O_3$ into the proteins was also markedly depressed in the presence of unlabelled glucose. Incubation of 300 mg. packed mammary carcinoma ascites cells (obtained from the same animals as used for exp. No. 1 of Table IV) with 32 μC $NaHC^{14}O_3$ (0·45 mg.) yielded proteins containing 100 counts per minute at " infinite " thickness, whereas in the presence of unlabelled glucose (36 μmole) the radioactivity amounted to 25 counts per minute. By carboxylation of pyruvate bicarbonate is fixed into oxaloacetate (→ aspartate) and the oxaloacetate-4-C^{14} thus formed is converted into α-ketoglutarate-1-C^{14} (→ glutamate). The tracer cannot reach glycolytic intermediates (formation of phosphopyruvate) since the C^{14} is set free by the decarboxylation of α-ketoglutarate.

TABLE V.—*Radioactivity of Glutamic and Aspartic Acid Derived from the Proteins of Ascites Tumour Cells Labelled by Acetate-1-C^{14}*

Amino acids isolated by ion exchange (Amberlite-IR 4 B), plated on 1·1 cm.² area and assayed at " infinite " thickness.

Tumour	Substrate	Protein	Glutamic acid	Aspartic acid
Rhabdomyosarcoma	A*	279	1080	725
	A*+G	70	291	225
Mammary carcinoma	A*	136	423	288
	A*+G	57	181	99

The radioactivity appearing in the respiratory carbon dioxide of the experiments of Table IV was also measured and it was found that in the presence of glucose the oxidation of DL-leucine-1-C^{14} was reduced to 20 per cent of that observed in the absence of glucose. In the breakdown of leucine-1-C^{14}, acetyl (-1-C^{14})-coenzyme A is formed and the labelled 2-C fragments are oxidized via the citric acid cycle to $C^{14}O_2$. The latter process is not impaired in the presence of glucose as was shown above. However, in the chain of reactions leading from leucine to acetyl-coenzyme A, the sequence :

$$\text{isovaleryl-coenzyme A} \xrightarrow{(-2H)} \beta\text{-methylcrotonyl-coenzyme A} \xrightarrow{(H_2O)} \beta\text{-hydroxy-valeryl-coenzyme A}$$

is essentially of the type found in the fatty acid oxidation cycle (Coon, Robinson and Bachhawat, 1955). The latter reaction may even be catalyzed by crotonase. Since the fatty acid oxidation cycle is impaired in the presence of glucose, the possibility that the same situation is also responsible for the impairment in the breakdown of leucine at the dehydrogenation step may be envisaged.*

In contrast to the oxidation of leucine, the oxidation of DL-glutamate-1-C^{14} by the mammary carcinoma cells was markedly enhanced in the presence of glucose (200–250 per cent, Table IV).

These experiments were carried out with exceedingly small concentrations of highly labelled amino acids in order not to disturb the pool sizes of the free amino acids, and, consequently, the metabolic equilibria of the type α-keto acid $\rightleftharpoons$ α-amino acid. The latter reaction involving :

$$\alpha\text{-ketoglutarate} + NH^+_4 + DPNH \rightleftharpoons \text{L-glutamate} + DPN^+ \quad . \quad . \quad . \quad (1)$$

and coupled with transamination reactions (L-glutamate + α-keto acid $\rightleftharpoons$ L-α-amino acid + α-ketoglutarate), is of vital importance in amino acid metabolism, since the equilibrium of reaction (1) decides whether amino acids are formed or broken down.

Since glucose appears to shift reaction (1) to the left, it may be concluded that less glutamate is formed from the citric acid cycle intermediate α-ketoglutarate in the presence than in the absence of carbohydrate. Consequently transamination reactions, *e.g.* leading to aspartate, are also slowed down. This interpretation may account for the decreased incorporation of C^{14} from acetate-1-C^{14} into the amino acids of the protein brought about by glucose. The latter effect, as pointed out above, is, at least as far as our experiments go, characteristic of the ascites tumour cells ; no such effect has ever been consistently observed in this laboratory with a solid tumour. In accordance herewith stands the finding that the oxidation of glutamate by slices of various solid tumours was depressed or not markedly affected in the presence of glucose, whereas the oxidation of leucine was inhibited but to a far lesser extent than in the case of the ascites cells.

Why does glucose impair the formation of glutamate in the ascites cell ? This question can only be answered in a speculative manner at present. Considering

* This conclusion is valid if the transamination reaction leucine + α-ketoglutarate $\longrightarrow$ α-keto-isocaproate + L-glutamate is not impaired by a lack of α-ketoglutarate (see below). The effect of unlabelled glucose on the pattern of incorporation into the proteins and of the oxidation of DL-lencine-1-C^{14} was similar for the two ascites tumours. However, glucose moderately depressed (30 per cent) the incorporation of DL-glutamate-1-C^{14} into the proteins of the rhabdomyosarcoma cells. The oxidation of glutamate by the latter cells in the presence of glucose was 1·5 fold that observed in the absence of carbohydrate.

(1) as the key-reaction, carbohydrate catabolism may lower the steady-state concentrations of either α-ketoglutarate or DPNH as compared with the levels reached during fatty acid catabolism. The protein sparing effect of carbohydrate in normal liver has been explained (Miller, Burke and Haft, 1955) on the basis that an active glycolysis enhances the DPNH/DPN^+ ratio and thus counteracts amino acid breakdown. This interpretation is exactly opposite to that given to explain the situation in the ascites cell.

Further experiments are being carried out on these points.

SUMMARY

The oxidation of acetate-1-C^{14} by ascites cells of a rhabdomyosarcoma and a mammary carcinoma is hardly, if at all, depressed in the presence of excess unlabelled glucose.

Since the oxygen consumption is markedly less under the latter condition (up to 50 per cent) it is concluded that the oxidative degradation of endogenous fatty acids to their 2-C fragments, but not the passage of these fragments through the citric acid cycle, is primarily inhibited as a result of the glucose supplementation.

Glucose favoured the incorporation of acetate-1-C^{14} into the long-chain fatty acids, but depressed the incorporation of tracer from acetate-1-C^{14} or $HC^{14}O_3^-$ into the trichloroacetic acid-insoluble proteins.

The incorporation of DL-leucine-1-C^{14} or DL-glutamic acid-1-C^{14} into the proteins was not affected by glucose addition but the oxidation of leucine was inhibited whereas that of glutamate was enhanced. The latter observations may serve to explain the depressive effect of glucose on protein labelling by acetate-1-C^{14} which was found to be characteristic for the ascites form of the present tumours.

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THE HAEMOLYSINS OF NORMAL AND MALIGNANT RAT TISSUES

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NON-SPECIFIC tissue haemolysins have long been known to occur and the haemolytic activity of normal tissue extracts has been extensively investigated in attempts to explain normal *in vivo* red cell destruction (Maegraith, Martin and Findlay, 1943 ; Laser, 1950 ; Ponder, 1951, 1952*a*). Comparison of the findings from the majority of these studies is extremely complicated, however, because of the use of a diversity of tissues and extraction procedures. Thus haemolysins have been isolated in isotonic sodium chloride solutions either from freshly prepared or pre-incubated tissue slices or minces (Weil, 1907 ; Maegraith, Martin and Findlay, 1943 ; Ponder, 1951), whilst others have used water miscible or immiscible organic solvents such as ethanol and ether in the extraction procedures (Singer, 1940 ; Laser, 1950 ; Ponder, 1952*b*). The haemolytic activities of extracts prepared under such a variety of conditions obviously are not comparable and in many cases, the haemolysins bear little or no relationship to one another. In general, however, the water soluble haemolysins are less active than the lysins extracted with ethanol or ether and it has been suggested that they exist as weakly active lysin-inhibitor complexes (Ponder, 1951 ; Tyler, 1951).

Haemolytic material has also been isolated in fresh saline extracts of a number of malignant tissues, particularly spontaneous, and transplanted mouse tumours (Gross, 1948, 1949 ; Adelsberger, 1951 ; Ponder and Nesmith, 1952). Little is yet known about the nature or properties of these haemolysins and considerable disagreement still exists concerning their specificity. Thus, for example, Gross (1948, 1949) claimed that haemolytic activity was a specific property of malignant tissues since species-specific haemolysins could be isolated from mouse and human tumours but not from their normal tissues. In contrast, haemolysins have been isolated under comparable conditions from normal adult and fœtal guinea-pig tissues (Tyler, 1951) and normal mouse lung (Ponder and Nesmith, 1952). Comparison of the data obtained in these studies, especially that of Ponder and Nesmith (1952), reveals, however, that malignant tissue extracts generally have an increased haemolytic activity.

The survival of red cells from normal donors transfused to cancer patients has been extensively investigated during recent years (Sheets, Hamilton, De Gowin and Janney, 1954 ; Hyman and Harvey, 1954 ; Miller, Chodos, Emerson and Ross, 1956). These studies have shown that the life span of normal red cells is shortened on transfusion into cancer patients and it has been proposed that a humoral haemolytic factor plays an important role in the pathogenesis of anaemia in cancer (Hyman and Harvey, 1954). More recently, Hyman, Gellhorn and Harvey (1956) have shown that red cells from cancer patients have normal or near normal life spans when transfused into normal subjects indicating that there is no

intrinsic defect in the red cell and so further supporting the hypothesis of a circulating haemolytic factor. Whether this haemolytic factor and the increased haemolysin content of malignant tissue are related is still a matter for speculation. At present, little is known about the nature of tissue haemolysins whilst it still has to be shown that they can cause *in vivo* haemolysis. The present paper is concerned with methods of isolation and assay of tissue haemolysins and a comparison of the haemolytic activities of normal and malignant rat tissue extracts. Studies on the chemical and physical properties of rat tumour haemolysin will be described in a later publication.

MATERIALS AND METHODS

Tumours

Transplanted rat sarcomata originally induced in inbred Wistar rats with methylcholanthrene (Baldwin, 1955) were used as source of tumour tissue in most of the experiments. These tumours (S5, S66, S69) were used between the 1st and 37th generation of transplantation. Experiments were also performed with the Walker carcinoma and with two transplanted mouse tumours.

Preparation of tumour extracts

Initially when haemolysis tests were observed for periods up to 24 hours, tumour extracts were prepared under sterile conditions. Later, however, tests were observed for much shorter periods up to 5 hours and so no attempt was made to ensure that extracts were sterile. Extracts from single tumours were used for each test. The general technique utilized in the preparation of extracts was as follows: Tumour was collected as quickly as possible under aseptic conditions and transferred to an ice-cooled container. Portions of the healthy, peripheral section of the tumour were then homogenized in an ice-cooled, vertical top drive, blender (2–3 minutes at 13,000 r.p.m.) with 2 ml./g. wet weight of buffered isotonic sodium chloride solution (0·14 M NaCl, 0·01 M NaH_2PO_4, 0·01 M Na_2HPO_4, pH 6·8). Tumour homogenates were centrifuged immediately at 2000 G. and 5° C. and the supernatant fluids stored below 5° C. until tested for haemolytic activity.

Initially, extracts were prepared in unbuffered isotonic NaCl solution. The pH of these extracts varied considerably between pH 6·5 and 7·4 and later it was shown that haemolytic activity was pH dependent; the activity rapidly decreasing above pH 7·4. It was shown also that maximum haemolytic activity was obtained between pH 6·5 and 6·8 and so extracts were prepared in lightly buffered NaCl solution at pH 6·8. Extracts prepared in this medium had a pH range from 6·8 to 7·1.

Preparation of normal tissue extracts

Tests were performed on extracts of single organs and so the amount of tissue available in some cases was small. In addition, simultaneous tests were performed with extracts of several organs from a single donor and so high-speed homogenization was not used in the preparation of tissue extracts. Instead, organs were removed from the donor as quickly as possible and transferred to weighed, ice-cooled centrifuge bottles. The organs were then finely minced with scissors, extracted with buffered NaCl solution, pH 6·8 (2 ml./g. wet weight of tissue)

and centrifuged 2000 G. and 5° C. The haemolytic activity of the supernatant fluids was tested immediately following centrifugation.

Measurement of haemolysis

Red blood cells.—Blood of various species including rat, mouse, rabbit and guinea pig was taken under sterile conditions either from a vein or from the heart of anaesthetized animals and collected in heparinized isotonic NaCl solution (0·14 M NaCl, 100 International units Heparin/ml.) Red cells were collected by centrifugation (600 G. at 5° C.), washed three times with 0·14 M NaCl solution and diluted in this medium to give a 1 per cent v./v. suspension. Sheep red cell suspensions were prepared in a similar manner from sheep's blood obtained commercially. Red cell suspensions were always freshly prepared and used within 6 hours of preparation.

Haemolysis test.—The haemolytic activity of an extract was determined by measuring the time for complete haemolysis at 37° C. of a standard amount of red cells by various dilutions of the extract and constructing a time-dilution curve. From this curve, the activity of an extract was determined as the highest dilution producing complete haemolysis in a standard time (usually 5 hours).

The haemolysis test was performed as follows :

Serial dilutions of extract in buffered NaCl solution (0·2 ml.) were maintained at 37° C. $\pm$ 0·1° in a water bath and 1 per cent red cell suspension (0·1 ml.) at the same temperature was added to each tube at a known time. The red cells were thoroughly mixed with the extract and the time for complete haemolysis determined.

RESULTS

Extracts of rat tumours were weakly active against rat red cells so that haemolysis only occurred after incubation at 37° C. for 24 hours or longer (Table I). Rat red cells, however, were readily agglutinated and most extracts caused agglutination at dilutions of 1/32 following incubation at 37° C. for 5 hours. Only extracts of the Walker carcinoma showed any appreciable haemolytic activity for rat red cells and it should be noted that none of these extracts caused agglutination.

TABLE I.—*Haemolysis and Haemagglutination of Rat Red Cells by Extracts of Various Transplanted Tumours*

Tumour	Number of extracts tested	Number producing haemolysis and maximum dilution titres*		Number producing haemagglutination and maximum dilution titres*	
		5 hours	24 hours	5 hours	24 hours
Rat—					
Walker carcinoma	10	2 (32)	9 (32)	0	0
Sarcoma :					
S5	7	0	2 (4)	7 (32)	7 (64)
S66	5	0	2 (4)	5 (32)	5 (32)
S69	2	0	1 (4)	2 (32)	2 (32)
Mouse—					
Strain A :					
Mammary carcinoma	5	0	5 (4)	5 (64)	5 (128)

* Figures in parentheses indicate reciprocals of maximum dilution titres.

Rat tumour extracts were also haemolytic for the red cells of a sheep, rabbit and guiuea-pig. Again, however, the haemolytic activity was low so that extracts only produced haemolysis in one or two dilutions even after prolonged incubation.

In contrast, mouse red cells were very susceptible to haemolysis by rat tumour haemolysin and in many cases, complete haemolysis was produced by extracts at dilutions of 1/64 or greater following incubation for periods up to 5 hours. The source of the mouse red cells appeared to have little influence on their susceptibility to haemolysis and in experiments with mouse tumours, homologous red cells were haemolysed as rapidly as red cells from other strains of mice. Because of their susceptibility to tissue haemolysin, mouse red cells have been used in the haemolysis test system in most experiments since they permit haemolytic titres to be determined within a convenient period of time.

Haemolysis of mouse red cells by rat tumour extracts

Typical time-dilution curves for complete haemolysis of mouse red cells by rat tumour extracts are shown in Fig. 1 (*a*, *c*). The form of these curves reveals the presence of an inhibitory zone at high concentrations of extract so that on dilution,

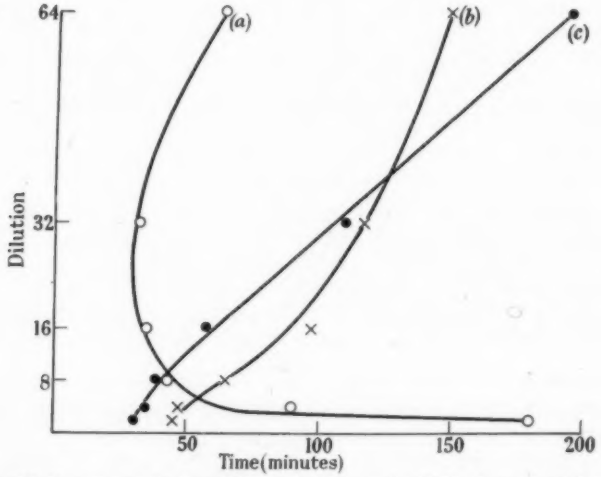

FIG. 1.—Time-dilution curves for haemolysis of mouse red cells by transplanted rat tumour extracts.

a and *c*.—Typical curves showing varying amounts of lysis inhibition at high concentrations of extract.

b.—Curve showing abnormal production of lysin during incubation of extracts.

the activity initially increases (haemolysis occurs more rapidly), passes through a maximum and then decreases with further dilution. Inhibition zones at high concentration were observed in all the tumour extracts tested although the amount of inhibition varied considerably. In many cases, considerable inhibition was obtained so that a definite maximum activity could be determined at some dilution of extract, usually in the range 1/4 to 1/16 (Fig. 1*a*). In other experiments,

slight inhibition was obtained in only one or two dilutions of extracts so that the inhibition zone appeared as a small inflexion on the time-dilution curve (Fig. 1*c*).

The inhibitory factor revealed at high concentrations of extract makes direct comparison of the activities of extracts extremely difficult. In previous studies (Tyler, 1951), the time required for complete haemolysis of red cells by a single arbitrarily fixed dilution of extract was used as a measure of haemolytic activity. Obviously this measurement does not necessarily measure the true activity of an extract since observations may be made in the inhibition zone. Determinations of the percentage haemolysis produced by various dilutions of extract after an arbitrarily fixed period of time are also unsatisfactory since the measurements may fail to reveal the inhibitory zone, whilst observations after prolonged incubation may be uncertain due to the production of fresh haemolysin from the incubated extracts (Ponder, 1951 ; Ponder and Nesmith, 1952).

The activity of extracts can be determined, however, by observing the maximum dilution of extract producing complete haemolysis at regular intervals over a period of time or preferably by determining the time required to produce complete haemolysis by dilutions of extract and constructing a time-dilution curve. If the measurements are made for a sufficient period of time, usually 3 to 5 hours, a maximum dilution titre can be determined whilst observations of the rate of haemolysis will indicate whether or not fresh haemolysin is being produced by pre-incubation effects. Thus the activity of an extract expressed as the maximum dilution producing complete haemolysis can be determined from the upper asymptote of the time-dilution curve whilst the form of the curve indicates whether or not lysin inhibitor is present and also whether pre-incubation effects are occurring. In several experiments, an increase in the slope of the time-dilution curve was observed after a certain period of time indicating an increase in the rate of haemolysis due to abnormal production of lysin (Fig. 1*b*). These results have not been recorded since interpretation of the curves is difficult.

The maximum dilution titres for haemolysis of mouse red cells by rat and mouse tumour extracts are shown in Table II. All of the transplanted rat tumour extracts were active against mouse red cells and the majority (72 per cent) had haemolytic titres of 1/64 or 1/128. Extracts of the two transplanted mouse tumours included

TABLE II.—*Haemolysis of Mouse Red Cells by Extracts of Various Transplanted Tumours*

Tumour	Number of extracts tested.	Range	Haemolytic titres.* Number of extracts showing titres of					
			4	8	16	32	64	128
Rat—								
Walker carcinoma	6	4–128	1	—	1	—	2	2
Sarcoma :								
S5	6	4–128	1	—	1	—	2	2
S66	15	32–128	—	—	—	4	7	4
S69	10	8–128	—	1	3	1	3	2
Mouse—								
Strain C :								
Sarcoma	9	4–64	2	1	3	1	2	—
Strain A :								
Mammary carcinoma	9	4–128	1	1	3	2	1	1

* Titres expressed as reciprocals of maximum dilution producing haemolysis.

for comparison had similar haemolytic titres although they were generally slightly less active.

Haemolysis by extracts of normal rat tissues

Normal rat organ extracts were feebly active against rat red cells so that haemolysis only occurred in dilutions up to 1/4 even after incubation at 37° C. for 24 hours or longer. The extracts showed considerably more activity against mouse red cells producing haemolysis at dilutions up to 1/64 following incubation for 3 to 5 hours (Table III).

TABLE III.—*Haemolytic Activity of Normal Rat Tissue Extracts Against Rat and Mouse Red Cells*

Organ	Number of extracts tested*	Number showing haemolysis at dilutions of: 1/2	1/4	1/8	1/16	1/32	1/64
(*a*) Rat red cells : 24-hour titre							
Liver	5/5	3	2	—	—	—	—
Lung	5/5	1	2	1	1	—	—
Kidney	1/5	1	—	—	—	—	—
Spleen	4/5	1	3	—	—	—	—
Heart	0/5						
Serum	0/5						
(*b*) Mouse red cells : 5-hour titre							
Liver	13/15	—	2	7	4	—	—
Lung	15/15	—	2	4	6	2	1
Kidney	14/15	—	3	3	8	—	—
Spleen	15/15	—	—	1	3	8	3
Heart	0/15						
Serum	0/15						

* Numerator—Number of extracts causing haemolysis. Denominator—Number of extracts tested.

All organ extracts, except those of heart tissue, possessed haemolytic activity, However, serum inactivated at 56° C. was completely inactive even after prolonged incubation. Heating at 56° C. did not destroy the activity of other tissue extracts. Spleen extracts possessed the greatest activity although in two experiments, lung extracts showed comparable activity. However, extracts of other organs were generally 2 to 4 times less active than spleen extracts. The time-dilution curves for complete haemolysis of mouse red cells by rat organ extracts (Fig. 2) reveal that in addition to being the most active, spleen extracts produce haemolysis more rapidly than any other organ extract. Thus, the maximum dilution of spleen extract showing activity produces haemolysis in a much shorter time than any of the other organ extracts at their maximum active dilution.

Inhibitory zones were again observed at high concentrations of organ extract although the amount of inhibition generally was much lower than that detected in tumour extracts. The amount of inhibition produced by various organ extracts also varied considerably, liver extracts showing the most and spleen extracts the least.

DISCUSSION

The results indicate that haemolysins can be isolated in saline extracts of normal rat tissues as well as from transplanted rat tumour tissue. These haemolysins are not species-specific since under the right conditions, they will haemolyse the red cells of several species. However, the mouse red cell appears to be very much more susceptible to this type of haemolysin than any of the other red cells tested. This may explain why in the earlier studies (Gross, 1948), it was claimed that mouse tumour haemolysin was species-specific, since with an insensitive technique for measuring haemolysis, red cells other than those of the mouse would appear to

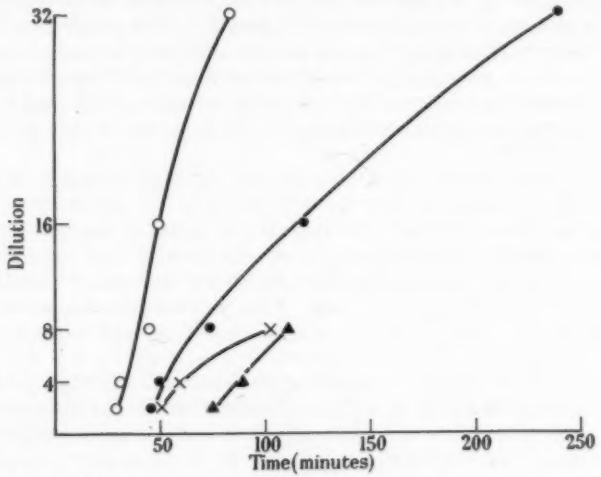

FIG. 2.—Typical time-dilution curves for haemolysis of mouse red cells by normal rat organ extracts.

Spleen ○—○
Lung ●—●
Kidney ×—×
Liver △—△

be resistant to the haemolysin. In fact the findings of Gross (1948) merely reflect the relative susceptibility *in vitro* of the mouse red cell to this particular type of haemolytic system. That varying red cell resistance series exist for different haemolysins is illustrated by the findings of Howard and Wallace (1953) who showed that the mouse red cell showed exceptional resistance to haemolysis by streptolysin O.

The relative resistance of rat red cells to haemolysis may be due to the fact that they are readily agglutinated by the tissue extracts. This would have the effect of making the red cells more resistant to the haemolysin. Haemagglutination activity has previously been observed by Salaman (1948) in extracts of a variety of tumours against mouse and rabbit red cells and by Gross (1948) and Ponder and Nesmith (1952) for rat and mouse tumour extracts against their homologous red cells. As suggested by Ponder and Nesmith (1952), agglutination and haemolysis

may be reciprocally related, the former occurring when the other does not and vice versa. It is perhaps significant that extracts of the Walker carcinoma which were much more haemolytic for rat red cells than any of the other rat tumour extracts tested, caused no haemagglutination.

In confirmation of the findings of Ponder and Nesmith (1952), atypical time-dilution curves were obtained for complete haemolysis of mouse red cells by both normal and malignant rat tissue extracts (Fig. 1 and 2), indicating the presence of an inhibitory factor at high concentrations of extract. This inhibitory factor makes direct comparison of the activities of extracts extremely difficult, The haemolytic activity of an extract can be expressed, however, as the maximum dilution producing haemolysis of a standard amount of red cells under fixed conditions of time and temperature, providing that no abnormal lysin production is occurring. This dilution titre will measure the maximum amount of haemolysin becoming available from the extract under the conditions used and will depend upon a number of competing factors such as the rates of inactivation of lysin and inhibitor and the rate of lysin production or activation from inactive or slightly active lysin complexes.

The haemolytic activities of various normal rat organ extracts were found to vary considerably ; spleen having the highest activity whilst serum and heart extracts were inactive. Studies of the rates of haemolysis by organ extracts indicated that in addition to being the most active, spleen extracts also produced haemolysis most rapidly indicating that the lysin was more readily available than that in other normal organ extracts. This may well be due to the absence of inhibitory substances since spleen extracts rarely showed inhibitory zones in time-dilution curves.

Comparison of the results obtained with extracts of normal and malignant rat tissue extracts (Tables II and III) indicates that the haemolytic activity of transplanted tumour extracts was generally 2 to 4 times greater than that of the most active normal tissue extract, i.e. spleen. Since the haemolytic system in rat tissue extracts consists of a mixture of lysins and inhibitors, the increased activity of tumour extracts may be due to an actual increase in lysin or to a decrease in inhibitor concentration. Although no quantitative data have been obtained regarding the variations of inhibitor, comparison of the time-dilution curves for haemolysis by extracts of normal organs; particularly spleen, and transplanted tumours indicated that the more active tumour extracts also showed the most inhibition. Thus, it would seem that there is an actual increase in the haemolysin content of tumour extracts.

Attempts to obtain evidence relating tumour tissue haemolysins to a humoral haemolytic factor so far have been unsuccessful since sera from tumour-bearing animals have not shown any *in vitro* haemolytic activity. This is not surprising however, since in the majority of previous studies on normal serum haemolysins, active extracts could only be prepared in non-aqueous solvents and it was suggested that lysin-inhibitors may be responsible for the *in vitro* inactivity of whole serum (Ponder, 1952*b*). These inhibitory substances are highly active *in vitro* and can inhibit the activity of a variety of haemolysins, many of which are far more active than the naturally occurring tissue haemolysins (Ponder, 1952b ; Stollerman, 1954). Therefore, it will be necessary to separate the naturally occurring serum lysins from the inhibitors before any conclusive evidence can be obtained regarding the relationship between serum and tissue haemolysins.

SUMMARY

1. Extracts of normal rat organs and transplanted tumours have been shown to contain a water soluble haemolytic factor. These haemolysins were not species-specific since they produced haemolysis of the red cells of several species including rat, mouse, rabbit, guinea-pig and sheep. However, the mouse red cell was very much more susceptible to the haemolysins than any of the other red cells tested. Extracts were only weakly haemolytic against homologous rat red cells although they readily caused haemagglutination.

2. Abnormal time-dilution curves for haemolysis of mouse red cells were obtained with extracts of both normal and malignant rat tissue extracts, indicating the presence of an inhibitory zone at high concentrations of extract. It is concluded that the haemolytic system consists of a mixture of lysins and inhibitors.

3. The haemolytic activity of normal tissue extracts varied considerably; spleen showing the greatest activity whilst heart extracts and serum were inactive. The haemolytic system in spleen extracts appears to be different from that of other normal tissue extracts. This is probably because spleen extracts contain little or no lysin inhibitor.

4. The haemolytic activity of transplanted tumour extracts was usually 2 to 4 times greater than that of the most active normal tissue extract, i.e. spleen. This increase in activity is probably due to an increase in haemolysin content rather than to a decrease in inhibitor.

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